THE ROLE OF LONG-CHAIN POLYUNSATURATED FATTY ACIDS AND RESVERATROL IN BRAIN NUTRITION: FROM BRAIN DEVELOPMENT TO AGING

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Nutrients play a key role in central nervous system (CNS) development during fetal and early postnatal life, and their depletions can have devastating physiological results. One class of nutrients these nutrients are long-chain polyunsaturated fatty acids (LCPUFA), specifically docosohexaenoic acid (DHA) and arachidonic acid (ARA). These lipids accumulate rapidly in the CNS, and although neonates are able to produce them from precursors at low rates, optimal development depends on preformed sources from human breast milk or formula. We report a descriptive meta-analysis that considered 106 studies of humauin breast milk and found that mean (±SD) concentration of DHA in breast milk (by weight) is 0.32 ± 0.22% and that of AA is 0.47 ± 0.13%. This comprehensive analysis of breast-milk LCPUFA indicates a broad range of these lipids worldwide and serves as a guide for infant feeding.

Dietary components with unknown or potentially deleterious effects remain important to characterize. While epidemiological data has implicated the role of trans fatty acid in increasing risk factors for heart disease, studies show that isolated trans fatty acids do not show atherogenic effects and remain difficult to accurately detect in mammalian tissues. We report these fatty acids in samples from patients with histologically-confirmed Alzheimer's disease and normal aged subjects and show differences to be nonsignificant. The

distributions of these trans fatty acids are consistent with their origin from diets that are a composite of dairy and partially hydrogenated vegetable oil trans sources.

While a number of factors combine to result in decreased function and cognition in aging humans, the hypotheses that oxidation plays a major role in aging has garnered much attention. While many antioxidant-derived plant compounds have provided promise against aging in animal models, resveratrol, a grape polyphenol, has received significant attention for its antioxidant properties and action as a calorie-restriction mimetic. We fed dietary resveratrol to wild-type and transgenic Alzheimer's Disease mice and show a number of targets specific to the pathophysiology of Alzheimer's, specifically transthyretin, drebrin, and glycogen synthase kinase-3, are positively influenced. These data suggest new mechanisms whereby this polyphenol putatively exerts protective effects in aging and beyond.

BIOGRAPHICAL SKETCH

Behzad Varamini was born in Manhattan, Kansas to Maryam and Hossein. Behzad spent his childhood growing up in Kansas (enduring tornadoes), Montana (enduring geysers), and Wisconsin (enduring lactose). After moving to Pennsylvania, Behzad attended Elizabethtown College to study biotechnology. There, after hearing a seminar about how how broccoli could cure cancer (in a flask), Behzad became increasingly interested in the role of nutrition in disease and spent two summers at the Nutritional Science Research Group at the National Cancer Institute. This experience propelled him to apply to Cornell University for a PhD in Nutritional Science. Upon completion of his degree, Behzad has fulfilled his proper duty as an Iranian-American to earn a PhD, MD, or JD, and remains open about the future.

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The list of persons who have helped me, encouraged me, and pushed me along the way is tremendously long. Here are some people who stand out.

My advisor, Dr. J. Thomas Brenna, has modeled for me many aspects of what it takes to be a professional and successful scientist. His patience with me and openness to new ideas has benefited me greatly.

Dr. John A. Milner accepted me as a summer CRTA at NCI in 2003, which began and solidified my interest in human nutrition and metabolism. He was the critical factor in my discovering my own academic and scientific interests in the field of nutrition and encouraged me to study at Cornell.

My undergraduate advisor, Dr. Jane Cavender, continuously had faith in me and continued to give me opportunities to perform research as an undergraduate. She taught me the beginnings of molecular biology, phosphorylations, switches, and cancer. She continued to be an active researcher and mom and served as a great personal role model.

I have numerous friends and colleagues that also played instrumental roles in my development and my success. Pete Lawrence has aided me hugely in most aspects of almost every one of my projects while at Cornell. Everyone in the Brenna lab past and present has assisted with my development as a scientist.

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INTRODUCTION

1.1 Nutrition and the developing brain

Nutrients play a key role in regulating brain development during fetal and early postnatal life. Nutritional insults can have particularly pronounced effects between 24 and 42 weeks of gestation due to the rapid growth of a number of neurological structures and processes, including myelination and synapse formation during this period(1). While the young brain remains relatively plastic and able to recover from deficiencies after nutrition repletion, vulnerability remains as many depletions in critical stages of development result in irrecoverable developmental insults(2).

While all nutrients play important roles in neuronal development, a number appear to have great or more specific effects during late gestational or early neonatal periods. These include protein, iron, iodine, zinc, choline, and long-chain polyunsaturated fatty acids (LCPUFA).

Table 1.1 lists these important nutrients in early brain development, the primary function(s) of that nutrient in the brain, and the regions of the central nervous system (CNS) where the nutrient plays its critical role.

Table 1.1 shows that the effects of nutrient deficiencies are regionally distributed. During different developmental epochs, various regions of the brain undergo rapid development and nutrient requirements coincide with

growth spurts where demands for specific nutrients to support metabolic pathways and structural components are crucial. The hippocampus, visual and auditory cortexes, and striatim undergo rapid growth during late fetal and early neonatal life(2, 3). Myelination, the growth of electrically insulating material which wraps around the axon of a neuron, also accelerates during late fetal and early neonatal life and is subject to major setbacks if proper nutrients are not present. Though the field of brain nutrition remains relatively young, a careful balance and thorough understanding of CNS development and nutrient requirements is needed as a nutrient can at one point in development be necessary and at another toxic. For example, iron is a very selectively regulated nutrient in the brain whose excess or deficiency within a narrow range during different stages can induce abnormal brain development(4). Proper nutrition is also necessary to support and maintain non-neuronal and structural components of the CNS, such as glial cells.

Generally speaking, nutritional insults that occur early in developmental periods of any given process are more likely to have a greater effect on cell proliferation leading to changes in cell number(5). Conversely, deficiencies that occur later during the course of developmental processes affect cell differentiation, size, and synaptic connections and function. Studies in the developing rat have shown that postnatal day 7 serves as an important benchmark in brain development, before which cell proliferation is the predominant trend in the brain.

After postnatal day 7, a number of genes that control differentiation exhibit

Table 1.1. Nutrients and their deficiency in the brain by function and structure/processes affected.

Nutrient	Function of nutrient in brain during development	Processes or structures primarily affected by deficiency of nutrient
Protein (6)	Neuronal proliferation and differentiation, synaptogenesis	Cortex, hippocampus
Iron (7, 8)	Myelin formation, monoamine synthesis, neuronal energy metabolism	White matter, Frontal lobe, hippocampus
Zinc (9, 10)	DNA synthesis, neurotransmitter release	Autonomic nervous system, hippocampus, cerebellum
Choline (11)	Neurotransmitter synthesis, neuronal energy metabolism, antioxidant activity	Hippocampus, white matter
LCPUFA (12)	Synaptogenesis, neurotransmitter systems, myelin formation, memory, secondary messengers	Global, eye

preferential increases in expression(13). Although an imperfect model, it is worthwhile to note that postnatal day 7 in the rat brain roughly corresponds to late-gestation in the human fetal brain(14).

Aside from structural changes and connections, nutrition can also affect the chemistry of normal brain processes. Nutrition has been shown to play important roles in neurotransmitter synthesis and reuptake(15). Although these changes are largely transient compared to structural changes, research continues to shed light on the role of nutrients in early CNS development events.

1.2 n-3 Long-chain Polyunsaturated Fatty Acids

n-3 long-chain polyunsaturated fatty acids (LCPUFA) are essential components of cell membranes throughout the CNS and in the retina. Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are the most abundant LCPUFA in the developing human brain (Figure 1.1) (16). The synthesis of these LCPUFA in humans appears to occur in great variability depending upon single nucleotide polymorphisms in a series of desaturate and Elongase enzymes required for their synthesis from precursors(Figure 1.2) (17). However, the developing fetus remains largely dependent on the maternal supply of these fatty acids because their synthesis and conversion for precursors remains low.

Under conditions of adequate supply, LCPUFA accumulate rapidly during the last trimester of pregnancy and throughout the first two years of life and are concentrated in neuronal membranes. More specifically, DHA is highly concentrated in brain grey matter and retina rod photoreceptors(18). Retina membrane phospholipids are comprised of over 45% DHA, and approximately 14% of brain gray matter fatty acids are DHA.

Evidence from studies in a variety of mammalian species including humans shows that the developing brain responds to changes in the dietary fatty acid supply with changes in tissue fatty acid composition. In both humans and rodents, dietary n-3 fatty acid deficiency results in decreased DHA in brain phospholipids(19, 20).

High levels of DHA in the retina and brain have led to increased research about its functional effects and outcomes associated with visual and cognitive development. Studies exist for which human infants have been assigned randomly to be fed formula supplemented with DHA and ARA, and then tested by standardized developmental scales. Infants supplemented with ARA and DHA during the first postnatal months have shown a seven-point increase in the mental development index (MDI) relative to controls(21). These infants also showed enhanced visual maturation and a correlation between plasma red blood cell DHA at 4 months and Mental Development Index scores at 18 months. Further, a separate study showed at 10 months that infants fed for 4 months a formula containing ARA and DHA performed better in a well-controlled means-ends problem-solving task than controls(22). Strikingly,

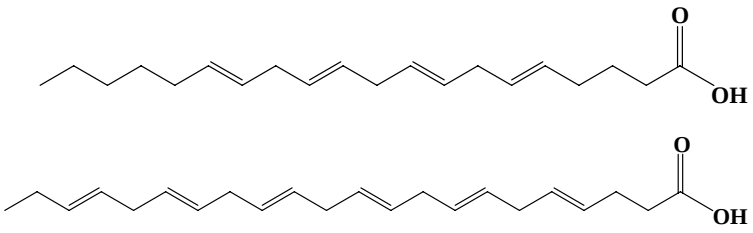

Figure 1.1. Chemical Structures of LCPUFA ARA (above) and DHA (below).

maternal supplementation with LCPUFA has also shown to result in significant increases in IQ at four years of age, demonstrating that n-3 PUFAs during pregnancy and lactation may be favorable for later mental development of children(23).

As such, it is clear that LCPUFA play a key role in human CNS development. Human breast milk LCPUFA are variable and reflective of maternal diet. While breast milk is considered the ideal source of nutrition, supplementation of infant formulas with increased preformed LCPUFA also represents an important avenue for delivery of these critical nutrients to the developing infant brain. Prior to 1995, infant formulas worldwide were devoid of LCPUFA. While the United States infant formulas have contained DHA and ARA since 2002, the amount of DHA and ARA required for optimal development has not been well characterized. In 2001, a group of clinical researchers recommended a minimum of 0.35% (w/w) DHA and 0.40% (w/w) ARA(24). The amount of DHA and ARA present in infant formulas varies (in the United states 0.32-0.35% DHA and 0.6% ARA in all formulas except one company which includes 0.15% DHA and 0.4% ARA). Benefits to term infants of increased DHA levels have not been thoroughly investigated. Further, while worldwide breast milk levels have a mean of about 0.32% (w/w), ranges of at least 1% have been described in Inuit women of northern Canada who average about 1.4% DHA in breast milk. At these levels of DHA and other n-3 LCPUFA, it has been shown that the lipids have specific bioactivities associated with cardiovascular function that are not observed at more moderate intakes.

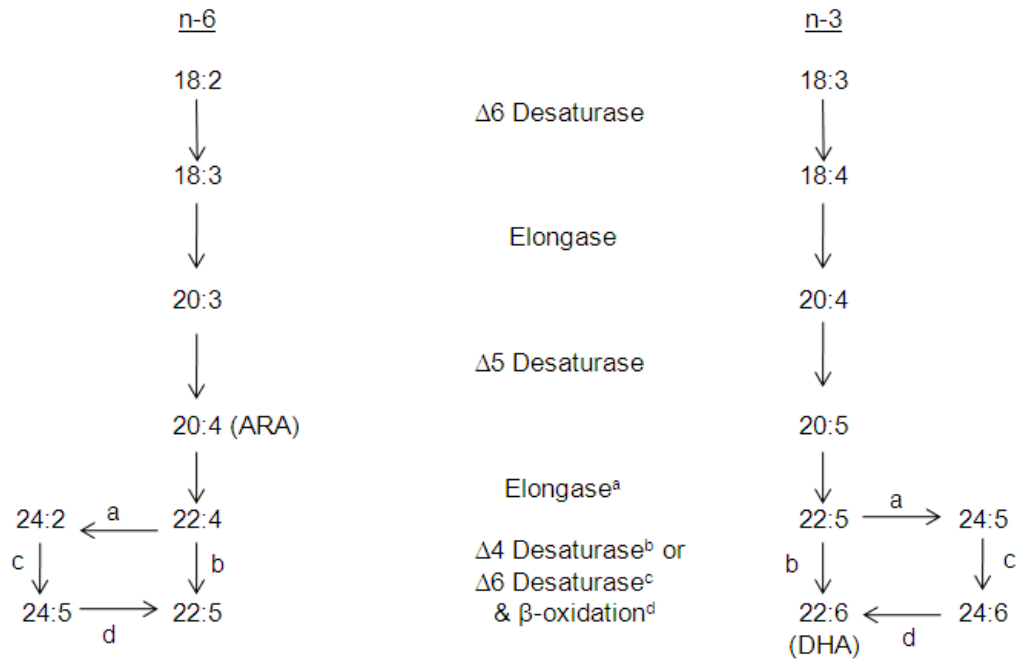

Figure 1.2. Metabolic pathways for conversion of linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3) into long-chain polyunsaturated fatty acids(18).

Strong evidence that increased levels of DHA may impart benefits comes from the work of Hsieh et al. who demonstrate that moderate to high levels of LCPUFA supplementation in baboon neonates results in cerebral cortex DHA increases(25). This data demonstrated that DHA at concentrations higher than presently used in formulas normalizes CNS tissue composition closer to that of breastfeeding. Further, DHA and ARA supplementation has been shown to enhance the oxygen carrying capacity of neonatal blood, suggesting that LCPUFA may alleviate the severity of neonatal anemia(26).

While increases in tissue concentrations and other biochemical parameters are compelling, these results should be combined with other studies of efficacy associated with improvements in functional outcomes. Until that time, it is reasonable to suggest that infant formula's supplemented with LCPUFA mirror worldwide breast milk averages which are known to be safe and effective.

1.3 Nutrients with unknown or potentially deleterious effects in the brain

While a number of nutrients demonstrate an important role in normal brain development and function, diet may also provide an avenue which leads to harm in the CNS, either at normal or average levels of intake, or through excess. Specifically, lipids, which constitute about 50% of the dry weight of the brain, represent an important macronutrient with a broad range of molecules with varying function and action in the CNS.

Saturated fats represents a significant portion of energy, be it from early in life through breast milk or formula, or later in life through any number of foods

such as meat, dairy products, and cookies and pastries. Epidemiological studies provided the first evidence that saturated fat may be linked to brain function. Specifically, two large Dutch prospective population-based studies have provided the most epidemiological information regarding this relationship. In the Rotterdam study, of more than ten thousand subjects of age 55 and older, persons were followed for three to four years and dietary intake information as well as mini-mental state examinations, or a test of brain function in aged subjects, was administered. After adjustment for a number of cofounders, total fat, saturated fat, and cholesterol intakes were shown to increase the risk of dementia, and total and saturated fat especially of dementia with a vascular component(27). The Zutphen elderly study consisted of 939 men followed for eight years and demonstrated increased total fat intake but not saturated fat intake was associated with impaired cognitive function(28). In contrast, studies in Spain demonstrate that increased consumption of saturated fatty acids is associated with decreased cognitive function(29). While the underlying mechanisms behind such a possible association are poorly understood, mechanistic evidence from animal studies has led several researchers to hypothesize that saturated fat leads to decreases in neuronal plasticity through reducing brain-derived neurotrophic factor(30) as well as decreased insulin sensitivity which is associated with decreased cognitive function(31, 32) among others(33). It is of great importance to note that not all saturated fats have equivalent effects, as lauric (12:0), myristic (14:0) and palmitic acids (16:0) have been shown to possess hypercholesterolemic properties as compared with oleic acid (18:0) in humans(34).

Trans fatty acids (TFA) have also been studied in the development and maintenance of normal cerebral function. TFA enter the food supply as products of chemical hydrogenation intended to destroy labile polyunsaturates to increase shelf life and to fine tune physico-chemical properties of oils in order to enhance the palatability of foods(35). Specific *trans* isomers also occur naturally as components of ruminant fats generated by bacterial biohydrogenation. Studies have shown that intake of industrial-derived TFA has been associated with several chronic diseases including coronary heart disease(36). Further, TFA have been associated with impairments in essential fatty acid metabolism, direct effects of which have been observed to alter cognitive function(33, 37).

Trans fatty acids have been blamed for many of the adverse effects associated with consumption of partially hydrogenated vegetable oil (PHVO). Specifically, PHVO *trans* 18:1 fatty acids, while variable, can comprise up to 50% of the fatty acids in PHVO, with trans-9 18:1 (elaidic acid) and trans-11 18:1 (vaccenic acid) as the most predominant isomers. However, as even all *trans* fatty acid isomers may offer different biological functions, it has remained unclear the potential contribution of each specific isomer in PHVO to coronary heart disease risk, as specific bioactivities had not been investigated prior to 2009. In 2009, a critical investigation directly compared these *trans* isomers with the effects of a PHVO diet in a Golden Syrian hamster model(38). In comparison to the control diet, the study found that the PHVO diet increased plasma ratios of total:HDL cholesterol and non HDL:HDL cholesterol by 17 and 23% respectively. Alternatively, the elaidic acid (trans-9 18:1) diet decreased these values by 27 and 46% and the trans-vaccenic acid (trans-11

18:1) diet decreased these values by 8 and 14% respectively, both statistically significant positive changes. To much surprise, these results indicate an improvement in markers of atherosclerosis risk by feeding elaidic and trans-vaccenic acid, while PHVO increased risk factors. Therefore, other factors present within PHVO are likely responsible candidates for increased coronoary heart disease risk. These data are particularly striking because a large pool of research has implied deleterious effects of TFA in PHVO in a number of tissues via numerous pathways(36), partially resulting in the labeling of TFA on foods sold in the United States and bans in many major US metropolitan restaurants in 2006. Overall, it is clear that much more research needs to be undertaken to specify the specific role of TFA and PHVO in human health.

Only a handful of research exists regarding TFA and its potential effect in brain development and early life. Pregnant rats fed diets with varying concentrations of *trans* fatty acids did show decreased levels of the essential fatty acids linoleic (18:2n-6) and α-linolenic (18:3n-3) but not LCPUFA(39). Further, newborn pups from mothers fed *trans* fat diets while pregnant exhibit decreased LCPUFA in plasma and liver but not brain of newborn pups(40). Dietary *trans* fatty acid fed to pregnant rats has shown to be associated with decreased activities of Δ6-desaturase in the liver, a critical enzyme involved in the pathway of endogenous LCPUFA formation from precursors in newborns(41).

Interestingly, during normal lactation in humans, *trans* fatty acids are present in human milk in high amounts and have been reported to be absorbed and stored in various tissues and organs(42) except brain(43). Such data has

caused Larque et al. to suggest that a protective mechanism to limit the incorporation of *trans* fatty acids in the CNS is in place(44). As far as the specific role of TFA in normal aged populations, only one study has suggested that dietary TFA intake is associated with diseases of the brain such as Alzheimer's disease (45). There are as yet no reports of TFA in the CNS, and no studies have been performed on the role of TFA in nervous tissue.

As measurements remain difficult to obtain, studying the potential action of this class of fatty acids or any specific isomer in the brain during normal maintenance and aging remain unclear. It is worth noting that a number of difficulties arise in studying TFA. Highly sensitive methods are required to measure these fatty acids and separate individual isomers from each other, especially as they exist in low concentrations in non-ruminant animals not consuming high amounts of PHVO. Chromatographic separation relying on older technologies has not always produced reliable results. In order that the individual bioactivities of isomers can be studied accurately, the development and implementation of highly sensitive and reproducible methods to detect TFA at low concentrations needs to continue to be an area of focus. It will also be important for researchers to keep in mind the potential effects of foodstuffs that contain TFA and their specific origins in food, such as in the case of PHVO versus natural (ruminant) sources. Further, since individual *trans* isomers are always consumed in tandem and never in isolation, studies examining the additive effects of TFAs in distributions normally found within the food system will need to be implemented as further research is done in brain.

1.4 Nutrition and the Aging Brain

As proper nutrition undoubtedly plays a role in normal health and development during all stages of life, special consideration must also be given to the aging individual. Aging represents a number of barriers and special challenges to the uptake and metabolism of nutrients to maintain health. The most widespread concern which affects most aged persons is acute or chronic digestive disorders. Past age 65, men and women are five times more likely to develop gall bladder disease, ulcers, and diverticulosis(46), all of which can lead to difficulties absorbing and digesting nutrients from food. Changes in taste and smell as well as difficulties swallowing and passing food influence intakes in older populations and show that dietary preferences change as one ages(47). Aside from practical and behavioral changes, biochemical changes in the body lead to subtle changes in metabolism and nutrient requirements for a broad range of vitamins and minerals in the elderly. Changes in gastric secretions and pH and decreases in lean body mass and chronic disease factors provide a myriad of variables to navigate in determining vitamin needs. Some evidence suggests that the current daily recommended intakes for vitamin D, vitamin B6 and B12, vitamin C, and folate may not be adequate for older persons(48).

As declining neurocognitive function presents as a major determinant to the quality of life for elderly persons, a large effort has been placed on discovering factors that slow or stop aging: the search for the supposed “fountain of youth”. While many assume that aging is simply a natural phenomenon that results in memory and cognitive decline, a batch of newer studies is beginning

to show that a large portion of the clinically significant decline in function in the elderly population can be averted or slowed(45). As such, dietary and pharmacological interventions have been evaluated to estimate their relative contribution to protecting from loss of mental function. While the role of non-modifiable factors, such as genetics and family history, are not trivial and still remains largely unknown, research interest in the causal and preventative roles nutrition and in aging has grown.

Almost all aged persons will exhibit some cognitive setbacks regardless of genetics or diet. Dementia describes a syndrome characterized by multiple cognitive deficits that lead to impairments in occupational and social functioning. Dementia is largely broken down into two discrete classes of illness: Alzheimer's Disease and vascular dementia(49). While the diseases present themselves differently pathologically, both have been shown to be responsive to dietary manipulation. Table 1.2 summarizes the results of several prospective studies where diet improves cognitive function. While epidemiological research clearly shows diet to play a role in the prevention of dementia, a large body of work remains to determine the mechanism and specific actions of dietary components.

1.5 Nutrition and Alzheimer's Disease

Alzheimer's Disease (AD) is the most common form of dementia, characterized by progressive memory losses as a consequence of neuronal cell death, neuritic plaques, and neurofibrillary tangles(53). As in almost any

Table 1.2. Prospective studies demonstrating positive effect of dietary compounds on dementia

Dietary component	Population	Result
Fish (intake expressed as meals containing fish per week) (27)	Rotterdam, Netherlands - 55+ years of age	Fish intake was associated with a slower rate of cognitive decline
Fruits and vegetables (intake assessed in weekly intervals through questionnaires) (50)	Across US - women 70+ years of age	Total vegetable intake significantly associated with less cognitive decline
Red wine consumption (expressed as glasses/day, highest cohort drank 3-4 glasses/day) (51)	France - 65+ years of age	3-4 glasses of red wine/d associated with lower relative risk of dementia and Alzheimer's disease
Whole grains (grains, cereals, bread) (52)	Poland - 55+ years of age	Whole grain consumption lower in population with Alzheimer's Disease

syndrome, genetic and environmental factors interact in the development of clinical disease. Through its precise cause is unknown, a number of risk factors are involved in AD onset such as age(54), mitochondrial defects(55), ApoE4 genotype(56), and diet(57). Current treatment options, namely pharmaceuticals, offer little protection against the disease and often have side effects.

Early onset Alzheimer's disease affects a small population of individuals primarily before the age of 60 and is associated with mutations in the presenilin 1 and presenilin 2 genes located on chromosome 14 and 1, respectively. In addition, mutations have also been described in the amyloid precursor protein on chromosome 21. Altogether, these mutations lead to malprocessing of the amyloid precursor protein to hallmark amyloid beta plaque characteristic of Alzheimer's. However, most AD is sporadic and late onset, and represents a complex combination of genetic and environmental factors.

The major characteristic pathology of AD are senile plaques, composed primarily of the 39-43 amino acid peptide amyloid-beta. Neurofibrillary tangles, hyperphosphorylated forms of the microtubule-associated protein tau, are also a hallmark pathogenesis. The current dominating hypothesis in the field describes accumulation of amyloid-beta lesions from proteolytic processing of amyloid precursor protein (APP) as the driving force to a cascade of neurodegenerative events due to the toxic effect of APP metabolism on neuronal integrity(58). A brief outline of the major events involved in amyloid

precursor protein metabolism and Alzeheimer's Disease can be found in Figure 1.3.

While the decline observed during AD involves multiple factors that influence several systems, the pathogenesis of the disease is still poorly understood. Much of the current research in the field has focused on environmental variables that influence AD, such as diet. The growing population of elderly in the United States has led to increased awareness and urgency to study the disease (Figure 1.4).

1.6 LCPUFA and Alzheimer's Disease

LCPUFA, a critical nutrient in brain development, also remains important in maintaining and protecting healthy brain function. While the mechanisms of action of LCPUFA are complex, research is slowly starting to untangle its roles in the normal and aging brain. Broadly, the primary effects of LCPUFA in aging can be put into three categories: membrane effects related to its relationship with rhodopsin, modulation of eicosanoid production, and its relationship to neurotrophic and apoptotic factors.

There is strong evidence that LCPUFA, specifically DHA, influences and alters rhodopsin function. Rhodopsin is a membrane protein present in rod outer segments and where it accounts for about 90% of the protein content and functions in a biochemical cascade leading to hyperpolarization and activation.

Membrane fatty acids alter the ability of photons to transform rhodopsin to its activated state. Animal studies show that declines of DHA of 50% in brain and retina have been associated with changes in neural function and visual acuity(59). Data in human infants suggest infants with higher red blood cell levels of n-3 LCPUFA demonstrate improved visual acuity at 4 months of age, further suggesting that LCPUFA are involved in visual maturation(60).

LCPUFA play an important functional role as precursors of eicosanoids, oxygenated 20-carbon compounds with important regulatory roles as modulators of cellular responses. While arachidonic acid is the main substrate for most eicosanoids, they can also be produced from eicosapentaenoic acid (20:5n-3) and DHAl; DHA can similarly be modified into a 22 carbon signaling molecule called docosanoids. Whereas most eicosanoids are involved in the regulation of the circulatory system, the primary prostaglandins (PGE), a subset of the eicosanoid family, have direct neural activity. The formation of PGE_2 and $PGE_2\alpha$ result in altered release of neurotransmitters norepinephrine and serotonin, as well as sedation and sleep patterns(61). Human infants born of mothers with higher plasma DHA levels are demonstrated more mature neonatal sleep-state patterning(62).

As a neurotrophic factor, DHA administered to newborn rat retinal cells has been shown to lead cells to survive and differentiate into photoreceptor cells, whereas cells with no supply of DHA eventually die by apoptosis(63). DHA has also been shown to be antiapoptotic when taken up and esterified by membrane phospholipids during insult or serum starvation(65, 66). Some of

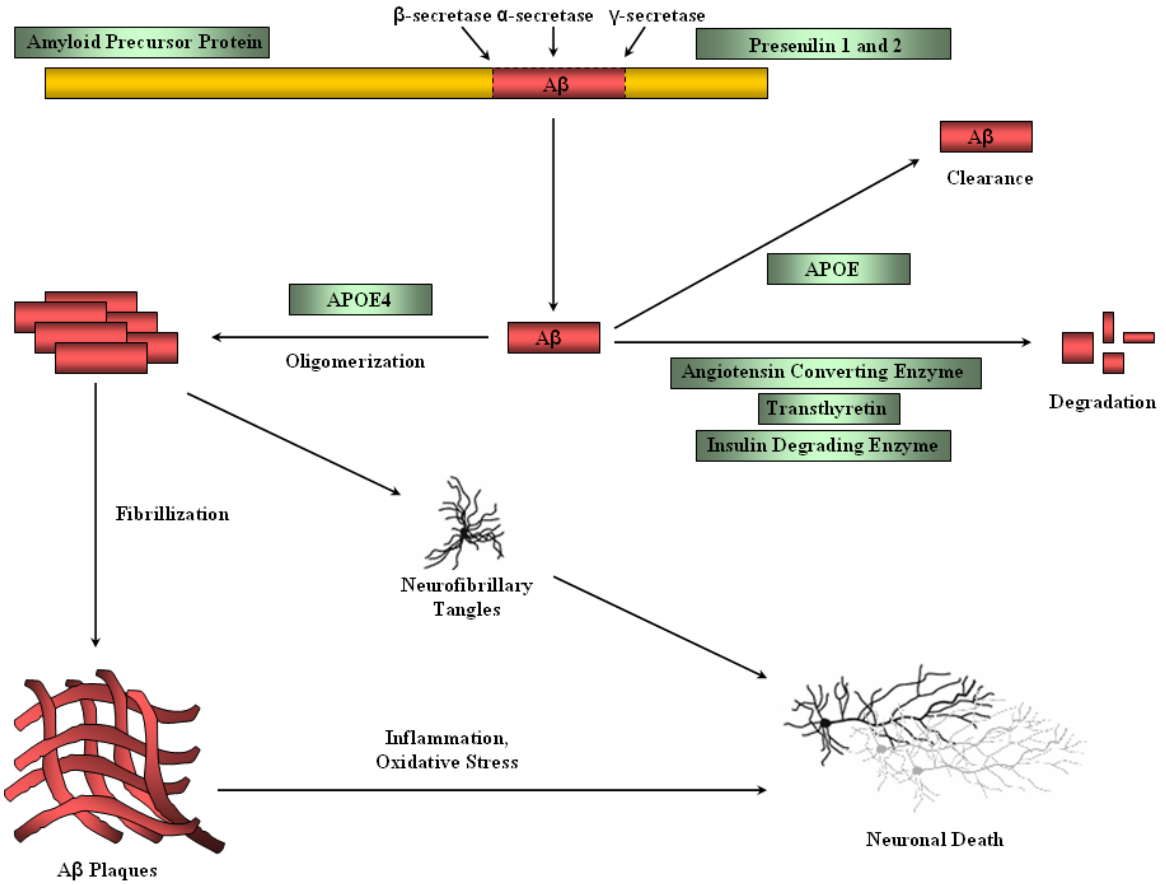

Figure 1.3. The main events associated with amyloid beta production in Alzheimer's Disease. Metabolism of APP by secretases can lead to Aβ oligomerization if the protein is not degraded. Plaques and neurofibrillary tanges have been associated with neuronal death.

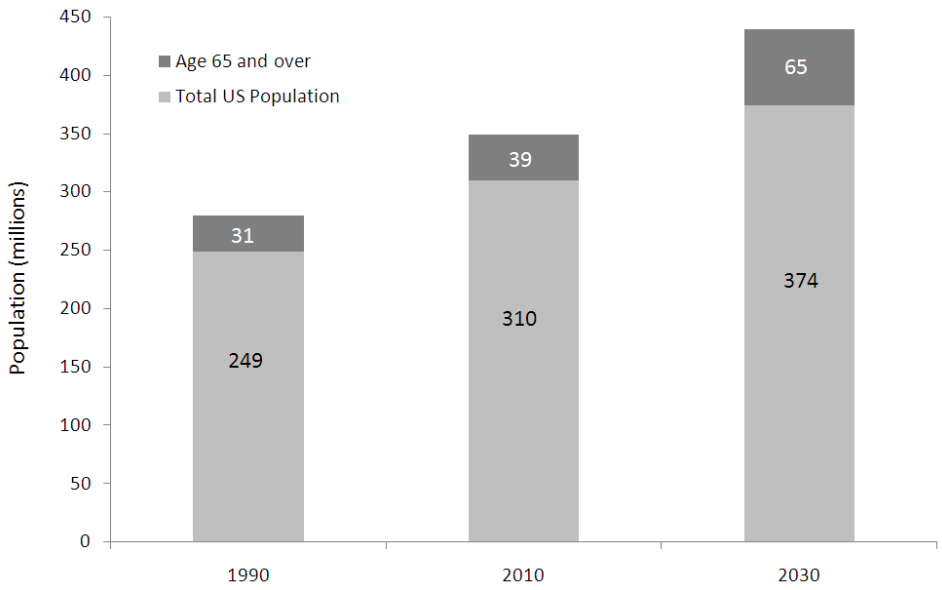

Figure 1.4. The growth of the older population in the United States, aged 65 and older. The population of the elderly has undergone tremendous growth and will continue at a rate higher than total population growth, a major consideration for diseases that appear later in life such as dementia and specifically Alzheimer's Disease. Data from Taeuber(64).

these actions, in part, are thought to be controlled by regulation at the transcriptional level, as PUFAs have been shown to bind and interact with peroxisomal proliferators activated receptor (PPAR) and hepatic nuclear factor 4-α(67).

In the aging brain, levels of DHA decrease and DHA is more susceptible to oxidation, leading to changes in nervous system function(68). Evidence supporting the importance of adequate LCPUFA levels in aging is growing, as decreased LCPUFA have been associated with increased risk of cognitive impairment(69). Mouse models have demonstrated that DHA gives rise to a compound known as neuroprotectin D1 which has been shown to be protective against post-stroke neuronal injury(70). A mouse model of Alzheimer's Disease demonstrated an association between decreased DHA in the frontal cortex with losses in key postsynaptic proteins involved in maintaining normal cognitive performance(71). One controlled trial in humans further demonstrated that higher fish intakes and LCPUFA consumption led to improvements in cognitive impairment in subjects with very mild Alzheimer's Disease(72).

1.7 Oxidation and antioxidants in Alzheimer's Disease

The free radical theory of aging, first proposed by Hartman in 1956, hypothesizes that the degenerative changes associated with aging may be a result of the accumulation of deleterious side reactions from free radicals produced during normal cell metabolism(73). The hypothesis suggests that free radicals can contribute to aging via several mechanisms:

1) Free radical induced DNA cross-links could lead to somatic mutations and loss of enzyme function
2) Oxidation of sufhydryl groups could cause cellular damage to microtubules
3) Membrane lipid peroxidation could destroy integrity of subcellular organelles

Since the hypothesis was conceived, considerable support has suggested and extended the notion that free radicals play an important role in the pathogenesis of neuronal degeneration. Specifically, the brain is a good substrate for oxidation because it is a large consumer of oxygen and polyunsaturated fatty acids, molecules highly susceptible to lipid peroxidation, are a major component of neural cell membranes.

Several studies indicate that elevated oxidative stress occurs in Alzheimer's Disease. First, AD brains exhibit significant increases in protein oxidation compared to age-matched controls(74). Similarly, 3-fold increases in mitochondrial DNA oxidation have been measured in the parietal cortex of AD brains compared to controls(75). Postmortem studies have shown increased lipid peroxidation in the frontal cortex of AD patients compared to controls(76).

Trace elements, such as iron, have been implicated to play a role in the generation of damaging radical oxygen species (ROS) in the AD brain. Iron and ferritin levels in AD are significantly increased in cortical gray matter regions, which facilitates the Fenton reaction and produces an abundance of ROS available for lipid perodixation(77) (Figure 1.5).

Further, dopamine catabolism is also a significant source of free radical generation(78). More specific to Alzheimer's disease, experimental evidence suggests that generation and aggregation of the amyloid beta protein produces increases in local ROS. In nerve cells, H_2O_2, a precursor molecule that is converted to the hydroxyl free radical, increased 3-fold after addition of amyloid beta to cell media(79). Data also suggests that AD patients have decreased CnZn-superoxide dismutase, glutathione peroxidase, and catalase in erythrocytes, three key enzymes that play a critical role in the normal health of a cell by fighting free radicals(80). Further, these enzymes are differentially downregulated in a number of regions of the AD human brain compared to controls(81).

One significant reason the oxidative damage hypothesis in aging has attracted considerable attention is because it may be potentially influenced by dietary antioxidants(76). Clinical and epidemiological evidence has found that the fat-soluble antioxidant vitamin E as well as the water-soluble vitamin C are significantly decreased in AD patients versus control despite adequate diets and are related to the degree of cognitive impairment(82, 83). In one randomized controlled trial, it was shown that α-tocopherol (vitamin E) delayed the occurrence of institutionalization, death, and loss of daily functioning and severe dementia(84). In another study, gingko biloba was evaluated in a group of AD patients and showed a modest advantage in supplemented patients on cognition, social functioning, and behavior(85), although one recent large review strongly suggested discontinued use of gingko biloba for prevention

and treatment of AD due to a number of side effects and very limited supporting data for its neuroprotection(86).

Clearly, there is a role for oxidative stress in the pathogenesis of AD. Beyond large scale studies evaluating the effectiveness of isolated antioxidants or dietary ingredients, molecular studies tailored to and describing the specific interaction between oxidation and Alzheimer's Disease have been quite telling.

As hypothesized by some researchers, plant-based compounds may offer additional benefits beyond simply providing additional antioxidants through vitamins(87). As many plant based products are considered safer than synthetic products, millions of Americans have turned to regular use of plant-based supplements to improve their health. While a number of these plant based compounds and their complete extracts have shown protection in neurodegenerative AD mouse models (Table 1.3), there is a high level of chemical complexity and many inherent difficulties in studying these products. The recent identification of more than 8000 phenolic compounds presents an awesome challenge to modern medicine and science(88).

Other than the sheer number of phenolic compounds, studying these biomolecules becomes increasingly complex since their mode of action and targets have been shown to differ in a concentration-dependent manner. For example, a low dose of red wine polyphenols has been shown to promote angiogenesis through activating the Akt/PI3K pathway but not the NF-κB pathway. Contrastingly, at higher doses, the polyphenols are anti-antiogenic and inhibit the Akt/PI3K pathway and enhance NF-κB signaling(89)

Similar is the case for epicatechin (a polyphenolic antioxidant found in cocoa, tea and grapes) where low concentrations have been shown to stimulate PI3K, an effect that disappears at ten-fold higher doses(90). Overall, these dose effects may be important factors in explaining the observed variability between the experimental outcomes in different models at varying concentrations.

1.8 Polyphenols in Alzheimer's Disease

The origins of research interest in red wine can be traced back to epidemiological studies that reported a low incidence of cardiovascular disease in the French, despite diets high in saturated fat. A popular and widely held theory called the "French Paradox" supposed that the anti-platlet aggregation properties of red wine components led to decreased atherosclerotic plaques(91). A significant number of studies have supported the notion that polyphenols and other components in red wine constituents demonstrate protective effects against neurodegenerative conditions and are reviewed here(92).

Briefly, phytochemicals can exert their protective health effects in a number of ways. Most obviously and first described, the wide variety of antioxidant molecules present in plants can scavenge oxygen and a number of other reactive oxygen species (ROS) in vitro, however the evidence that they significantly contribute to the antioxidant defense system in the central nervous system is not strong(93). Aside from their action as antioxidants, research suggests that polyphenols from foods may be more potent than

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

Figure 1.5. In the Fenton reaction, iron(II) sulfate interacts with hydrogen peroxide resulting in a hydroxy radical that is a biological oxidant.

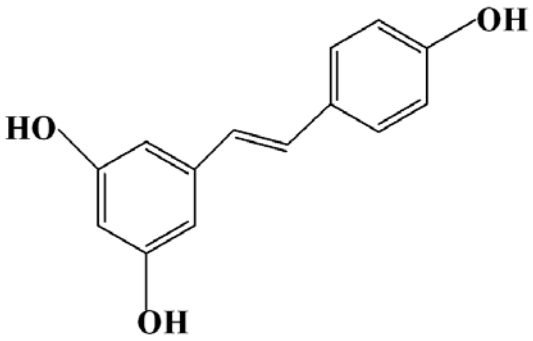

Figure 1.6. Resveratrol, a grape and red-wine polyphenol.

antioxidants administered as supplements(94). This has led researchers to hypothesize that these compounds may exert their activity by acting as signal transduction molecules or affecting the expression of genes. For example, the compounds resveratrol, curcimin and epigallocatechin gallate have been shown to block the activity of cyclooxygenase-2 and inhibit NFkB activation, important events involved in mediating the inflammatory response. Further, these compounds also stimulate the mitogen-activated protein kinease (MAPK) pathway, which leads to the activation of the antioxidant responsive element genes and a number of downstream detoxification enzymes(95). These plant-based compounds have also shown promise in altering behavior and neurocognitive ability in rats, as a number of studies have demonstrated that supplementation of mice with blueberries and strawberries led to improvements in spatial learning and memory as tested by Morris water maze(96). In addition, extracts from these fruits were further found to result in the reversal of age-related deficits in aged rats, including improved motor performance. The literature strongly suggests that polyphenolics, such as those contained in berry fruits, are a promising valuable asset in protecting and preventing development of age-related neurodegeneration.

1.8 Resveratrol in Alzheimer's Disease

While grapes contain a significant number of polyphenols, trans-resveratrol (3, 4', 5-trihydroxystilbene) has emerged as one of the most promising compounds (Figure 1.6). Resveratrol is also found in a number of other plants including peanuts, berries, as well as a popular Korean herb called kojo-jan(97, 98).

In vivo, resveratrol has proven more effective in protecting against oxidative damage than vitamins E and C combined(99). Resveratrol also protects against mutant polyglutamine-induced toxicity and neuronal degeneration (100). Further, the compound has demonstrated neuroprotective effects by alleviating neurotoxin-induced oxidative damage in cultured neurons(101). In addition, resveratrol has demonstrated anti-inflammatory responses by attenuating nitric oxide synthase and COX-2 expression; the activities of both play an important role in neurodegeneration(102, 103).

A number of *in vitro* studies have demonstrated resveratrol's neuroprotective ability specific to AD. Resveratrol markedly lowers the levels of secreted and intracellular amyloid beta peptides in several cell lines, as well as promotes intracellular degradation of amyloid-beta (104). The compound also restores glutathione levels, cell viability, and neuroplasticity *in vivo* (105). Lastly, resveratrol has been shown to improve mitochondrial function, organelles whose dysfunction is implicated in the pathophysiology of AD(106, 107). *In vitro* and *in vivo* evidence demonstrates resveratrol as CR mimetic, increasing sirtuin enzymatic activity and inducing a calorie restriction (CR) metabolic state (97, 108). Likewise, CR has also shown to be neuroprotective in several AD models(109-111), largely through a highly conserved sirtuin family (SIRT1, SIRT2, SIRT3) of genes. Sirtuin upregulation induced by CR mimetics has demonstrated effects on cell aging and AD risk as well as extending the lifespan of a number of species (112).

In vivo mouse models of AD have shown protective effects of fasting and calorie restriction, however, only a few studies to date have determined whether a CR-induced state by resveratrol or any other CR mimetic provides neuroprotection in a transgenic AD mouse model.

Of these studies, one demonstrated improvements in spatial-memory functions and decreased amyloidigenic peptides of AD transgenic mice consuming a Cavernet Sauvignon red wine for 7 months(97). In this study, it is interesting to note that improvements in cognitive function as well as biochemical changes did not accompany the ethanol only group, as some have hypothesized the beneficial effects of wine may be largely derived from its ethanol content. Previous studies have shown the positive effects of ethanol as alcohol consumption has been associated with decreased insulin resistance in humans as well as decreased body weight gain and liver triglycerides, and diabetes in mice(113, 114); experiments showing positive effects of ethanol on neurodegenerative conditions seems lacking and have at times shown mixed results which depend\ largely on concentration and timing of the ethanol dose(114). In contrast, experiments have also demonstrated the numerous positive effects of polyphenols on neuronal health and Alzheimer's diease transgenic mice, independent of alcohol, some of which can be found described in Table 1.3. Prominent scientists can be found on both sides of the issue. As studies in neurodegeneration comparing red wine constituents to ethanol are lacking, the debate regarding the partial benefit of alcohol or non-alcohol constituents of wine continues, and further work may be needed before a firm conclusion can be reached.

Table 1.3. Effect of numerous polyphenols and plants on AD mouse models

Polyphenol/Plant derivative	AD Model	Effects
Blueberry(115)	AD transgenic TG2576 mice	Improved Y-maze performance and decreased amyloid beta plaque burden
EGCG/Green Tea(116)	AD transgenic TG2576 mice	Decreased amyloid beta levels
Garlic(117)	AD transgenic TG2576 mice	Decreased amyloid beta levels, inflammation, and tangle-associated proteins
Ginseng(118)	AD transgenic TG2576 mice	Decreased amyloid beta levels
Pomegranate(119)	AD transgenic TG2576 mice	Improved water maze performance and decreased amyloid beta plaque burden

Further studies have shown resveratrol's potential protective capacity in neuronal health. Reveratrol supplementation alone reduced neurodegeneration and cognitive decline in mice expressing a coactivator of cyclin-dependent kinase 5 and displaying massive forebrain degeneration with AD features(66). In a seperate study, resveratrol was shown to reduce plaque pathology in the cortex of AD mice(18). While a number of mechanisms for resveratrol's protective effects in AD have been proposed, further identification and elucidation of targets are needed(120).

While much of the initial effort and thrust for resveratrol began after its modulation of Sirt1 was discovered, a number of studies have demonstrated that some of resveratrol's most important targets may in fact be Sirt-independent, potentially opening up the door to a broad range of pleiotropic action for the nutrient in Alzheimer's disease and other physiological conditions(121, 122).

1.10 Summary

LCPUFA represent a critical class of nutrients for optimal maturation of the CNS during neonatal and early life and a number of studies have examined different concentrations of LCPUFA in the development of neonates. This thesis examines worldwide breast milk DHA and ARA concentrations as a meta-analysis which can be used as a guide to infant feeding. *Trans* fatty acids have blamed for the negative health effects associated with partially hydrogenated vegetable oil consumption. While a number of studies have examined the physiological effect of *trans* fatty acids, these lipids remain

difficult to measure and haven't been reported in the human central nervous system, where mechanisms limiting their incorporation is hypothesized. Described herein is a highly sensitive method applied to normal aged and AD postmortem brain samples showing no differences between disease states and an envelope of isomers similar to a composite of dietary *trans* from hydrogenated oils and rumimants. Finally, researchers have focused on the use of antioxidants from plant compounds as potent antiaging agnets. We describe a study that examines the role of dietary resveratrol in a transgenic AD mouse model with focus on targets associated with amyloid beta degradation, post-synpaptic integrity, and tau pathology, demonstrating the broad effects of this molecule in neuroprotection and beyond.

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CHAPTER 2

DOCOSAHEXAENOIC AND ARACHIDONIC ACID CONCENTRATIONS IN HUMAN BREAST MILK WORLDWIDE*

2.1 Introduction

Human breast milk is universally recognized as the optimal food for term infants. Fat is a critical component of breast milk, providing energy and, importantly, nutrients key to the development of the central nervous system which cannot be synthesized de novo by the infant(1). Principal among these are the long chain polyunsaturated fatty acids (LCPUFA) docosahexaenoic acid (DHA) and arachidonic acid (ARA), which are now components of infant formulas in developed countries around the world. The synthesis of DHA and ARA from precursor fatty acids appears to be limited for at least some human infants(2, 3).

Both DHA and ARA are found in all breast milks examined to date using appropriate methodology. Short term diet clearly influences the LCPUFA content of breast milk and there is evidence that habitual intake has an influence as well(4-6). Fish eating populations have higher breast milk DHA concentrations than populations that do not consume marine foods(7, 8) and there is evidence that poorly nourished mothers conserve PUFA and LCPUFA in their breast milk at the expense of saturates(9). Breast milk fatty acid concentrations therefore vary with the lifestyle of the population of lactating

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mothers under study, and thus fatty acid concentrations vary by region.

The concentrations of human breast milk DHA and ARA have been reported since at least the 1970s(10). They have been tabulated in reviews (1) from small cross-sections of references, and these summary concentrations are quoted frequently. However, since breast milk DHA and ARA vary with diet, nutritional status, and other factors, analyses based on selected studies are biased because their findings are limited to the samples considered. There are no extant systematic reviews of breast milk DHA and ARA concentrations from the peer-reviewed literature.

Our goal is to establish the distributions of DHA and ARA concentrations in mature breast milks of free living mothers. Our strategy was to identify all papers in the peer-reviewed literature that report DHA and ARA concentrations in breast milks from mothers of term infants. Mothers must have consumed their normal diets that were not purposefully influenced by experimental manipulations, such as marine oil supplementation. From the database of all papers that were identified, we selected those that used modern capillary gas chromatography (GC) for analysis, capable of resolving DHA and ARA from compounds that elute nearby. We also included selection criteria related to the completeness of reporting and sampling. Summary statistics are provided for the main analysis group and the excluded group.

2.2 Subjects and Methods

Inclusion criteria. PubMed searches were performed with the keywords “breast

milk" and "docosahexaenoic" periodically from 2004, most recently in November 2006. Studies that were written in languages other than English were not included. All data were from mothers of term infants in good health consuming free-living or control diets during the intervention studies. Data from experimental groups who had special diets or consumed LCPUFA supplements were excluded in the primary analysis, as were experiments that analyzed pooled breast milk.

Studies that included data from only one mother, pooled or banked milk samples,and mothers of preterm infants were excluded. Because DHA and AA are more concentrated in phospholipids than are triacylglycerols, studies that reported concentrations by lipid class only were excluded. When values from multiple time points postpartum were available, the 2–6mopostpartum data were used. Studies meeting these criteria were split into 2 groups; the primary group consisted studies that used capillary GC columns that can fully resolve FA methyl esters with retention times very similar to those for DHA and AA; the secondary group consisted of mostly older studies that used packed GC columns which cannot resolve DHA and AA and thus may provide artifactually high values. We calculated means and SDs from both groups for comparison and reserved the analysis of the distribution of values for the primary group. FA concentrations are most often reported as a percentage of the total, by weight (wt:wt, or weight for weight). Several studies did not report FA data for saturates, monounsaturates, and PUFAs. Because percentages are the norm for reporting FAs, and percentages depend on the total number of FAs included in the calculation, we included only those values reported in the context of a full FA profile. All of the articles considered in this quantitative

Table 2.1. Studies included in the primary analysis[1]

Reference	Site	Infant age	Subjects	DHA[2]	AA[3]
		mo	*n*	*% of total fatty acids*[4]	*% of total fatty acids*[4]
Yuhas et al, 2006 (11)	Australia	1–12	48	0.23	0.38
Yuhas et al, 2006 (11)	Canada	1–12	48	0.17	0.37
Yuhas et al, 2006 (11)	Chile	1–12	50	0.43	0.42
Yuhas et al, 2006 (11)	China	1–12	50	0.35	0.49
Yuhas et al, 2006 (11)	Japan	1–12	51	0.99	0.4
Yuhas et al, 2006 (11)	Mexico	1–12	46	0.26	0.42
Yuhas et al, 2006 (11)	Philippines	1–12	54	0.74	0.39
Yuhas et al, 2006 (11)	United Kingdom	1–12	44	0.24	0.36
Yuhas et al, 2006 (11)	USA	1–12	49	0.17	0.45
Sala-Vila et al, 2008 (12)	Spain	0.5–1	10	0.31	0.49
Olafsdottir et al, 2006 (7)	Iceland	2	59	0.3	0.32
Xiang et al, 2005 (13)	China	3	23	0.18	0.51
Kovacs et al, 2005 (14)	Denmark	4	39	0.35	0.3
Jensen et al, 2005 (15)	USA, Texas	4	77	0.2	0.44
Bopp et al, 2005 (16)	USA. N.Carolina	3	22	0.21	0.41
Stoney et al, 2004 (17)	Australia	3	36	0.26	0.38
Sala-Vila et al, 2004 (18)	Spain	3	11	0.28	0.41
Minda et al, 2004 (19)	Hungary	1	18	0.19	0.59
Fraricois et al, 2003 (20)	USA, Oregon	2–11	14	0.2	0.5
Marangoni, et al, 2002 (21)	Italy	3	73	0.35	0.5
Krasevec et al, 2002 (22)	Cuba	2	52	0.43	0.67
Hawkes et al, 2002 (23)	Australia	1	27	0.26	0.46
Jorgensen et al, 2001 (24)	Denmark	4	39	0.35	0.3
Helland et al, 2001 (25)	Norway	3	111	0.47	0.37
Auestad et al, 2001 (26)	USA	4	29	0.15	0.48
Xiang et al, 2000 (27)	Sweden	3	19	0.25	0.38
Wang et al, 2000 (28)	Japan	0.3	20	1.1	1
Vander Jagt et al, 2000 (29)	Nigeria, Niger	0.3–6	34	0.2	0.51
Smit et al, 2000 (5)	Netherlands	3	25	0.14	0.33
Smit et al, 2000 (5)	Pakistan	12	8	0.06	0.26
Smit et al, 2000 (30)	Israel	3–10	10	0.15	0.49

(Continued)

Table 2.1 (Continued)

Reference	Site	Infant age	Subjects	DHA[2]	AA[3]
		mo	*n*	*% of total fatty acids*[4]	*% of total fatty acids*[4]
Okolo et al, 2000 (31)	Nigeria	0.1–0.5	28	0.32	0.58
Okolo et al, 2000 (31)	Nigeria	6–7	15	0.33	0.44
Marangoni et al, 2000 (32)	Italy	6	10	0.28	0.5
Knox et al, 2000 (9)	Nigeria, Niger	0.3–16	89	0.2	0.57
Jensen et al, 2000 (33)	USA	2	6	0.19	0.53
Fidler et al, 2000 (34)	Germany	1.5	5	0.21	0.43
Xiang et al, 1999 (35)	China	1	18	0.33	0.63
Makrides et al, 1999 (36)	Australia	4	33	0.2	0.39
Dodge et al, 1999 (37)	Xichang, China	2–18	10	0.22	0.52
Dodge et al, 1999 (37)	Beijing, China	2–18	10	0.28	0.63
Dodge et al, 1999 (37)	Enshi, China	2–18	9	0.15	0.35
Woltil et al, 1998 (38)	Netherlands	>0.3	29	0.19	0.4
Yu et al, 1998 (39)	Sweden	6	17	0.18	0.34
Rueda et al 1998 (40)	Spain	0.5–1	8	0.38	0.69
Rueda et al, 1998 (40)	Panama	0.5–1	8	0.32	0.52
Rocquelin et al, 1998 (41)	Congo	5	102	0.55	0.44
Maurage et al, 1998 (42)	France	1.5	15	0.14	0.24
Helland et al, 1998 (43)	Norway	0.75–2	22	0.38	0.34
Francois et al, 1998 (44)	USA	6	7	0.2	0.4
Innis et al, 1997 (45)	Canada	3	56	0.2	0.5
Billeaud et al, 1997 (46)	France	NR	25	0.32	0.52
Auestad et al, 1997 (47)	Canada	4	43	0.12	0.51
Ratnayake et al, 1996 (48)	Canada	0.75–1	198	0.14	0.35
Makrides et al, 1998 (49)	Australia	3	12	0.21	0.41
Jorgensen et al, 1996 (50)	Sweden	4	14	0.53	0.44
Huisman et al, 1996 (51)	Netherlands	3	25	0.19	0.34
Presa-Owens et al, 1996 (52)	Spain	0.6–1	40	0.34	0.5
Cherian et al, 1996 (53)	Canada	NR	5	0.3	0.4
Makrides et al, 1995 (49)	Australia	4	23	0.21	0.4
Luukkainen et al, 1995 (54)	Finland	3	10	0.18	0.33
Chardigny et al, 1995 (55)	France	0–3	10	0.32	0.5
Luukkainen et al, 1994 (56)	Finland	4	16	0.18	0.33

(Continued)

Table 2.1 (Continued)

Reference	Site	Infant age	Subjects	DHA[2]	AA[3]
		mo	*n*	*% of total fatty acids*[4]	*% of total fatty acids*[4]
Innis et al, 1994 (57)	Canadian Arctic	1–7	5	1.4	0.6
Innis et al, 1994 (57)	Vancouver	2–4	12	0.4	0.7
Budowski et al, 1994 (58)	Israel	1.5–2.5	26	0.38	0.59
van Beusekom et al, 1993 (59)	Netherlands	0.5–1	5	0.26	0.47
van Beusekom et al, 1993 (60)	Dominican Republic	0.75	7	0.4	0.5
Martin et al, 1993 (61)	France	1	24	0.24	0.36
Guesnet et al, 1993 (62)	France	3	28	0.38	0.5
Henderson et al, 1992 (63)	USA, Connecticut	0.5	5	0.37	0.67
Ogunleye et al, 1991 (8)	Nigeria	2–3	20	0.34	0.56
Ogunleye et al, 1991 (8)	Japan	2.3–3.3	53	0.53	0.36
Boersma et al, 1991 (64)	Saint Lucia	1	12	0.53	0.58
van Beusekom et al, 1990 (65)	Dominican Republic	>0.3	6	0.91	0.33
van Beusekom et al, 1990 (65)	Belize	>0.3	6	0.21	0.44
van der Westhuyzen et al, 1988 (66)	Urban south Africa	6.8	12	0.2	0.6
van der Westhuyzen et al, 1988 (66)	Rural south Africa	6.5	18	0.1	1
Koletzko et al, 1988 (67)	Germany	3–4	15	0.22	0.36
Innis et al, 1988 (68)	Canada	>3	17	0.2	0.5
Muskiet et al, 1987 (69)	Tanzania	>0.3	11	0.27	0.6
Muskiet et al, 1987 (69)	Curao	>0.3	47	0.43	0.71
Muskiet et al, 1987 (69)	Suriname	>0.3	20	0.41	0.58
Carlson et al, 1986 (70)	USA	0.5	11	0.19	0.59

1 A total of 84 studies including a total of 2974 subjects are reported.
NR, not reported; DHA, docosahexaenoic acid, AA, arachidonic acid.
2 Mean ± SD: 0.32 ± 0.22
3 Mean ± SD: 0.47 ± 0.13
4 By weight

review are listed in Table 2.1 and Table 2.2.

Sixty-five articles providing 84 mean values from 2474 subjects reported analyses with capillary columns and were judged to provide sufficient detail to be included in the primary analysis group (Table 2.1).The 41 articles judged to be outside the stated criteria and assigned to the secondary group are listed in Table 2.2.

2.3 Results

The distribution of DHA and AA concentrations (wt:wt) are shown in Figure 2.1, and the summary statistics are shown in Table 2.3. The mean (±SD) concentrations of DHA and AAin the primary analysis group were 0.32 ± 0.22% and 0.47 ± 0.13%, respectively. The secondary analysis group yielded somewhat greater values for DHA of 0.40 ± 0.41% and for AA of 0.56 ± 0.26%. The mean value for AA deviates by 0.09% (wt:wt) from that of the primary reference group, whereas the mean value for DHA deviates by 0.08% (wt:wt). These statistics are consistent with the hypothesis that the poorer resolution of packed-column GC yields higher values for DHA and AA than does capillary GC; these data also included a few studies with DHA and AA values from colostrum, which is considered richer in LCPUFA than mature milk. We conclude that our exclusion criteria yielded slightly lower overall mean LCPUFA concentrations. Considering only the primary analysis, the CV for DHA was 0.22/0.32 = 69%, whereas that for AA was 0.13/0.47 = 28%. SDs are a composite of 1) analytic error (including variability in sampling, extraction, derivatization, and signal processing) and 2) real biological

Table 2.2. Studies excluded from the primary analysis[1]

Reference	Reason for exclusion
Straarup et al, 2006 (71)	Preterm, pooled sample
Agostoni et al, 2003 (72)	Pooled sample
Lapillone et al, 2000 (73)	Pooled sample
Fidler et al, 2000 (34)	Analysis of colostrum
Schmeits et al, 1999 (74)	Analysis of milk TG only
Pugo-Gunsam et al, 1999 (75)	Analysis of milk TG only
Kaila et al, 1999 (76)	Banked samples
Guesnet et al, 1999 (77)	Few FAs reported
Bougle et al, 1999 (78)	Few Fas reported
Babin et al, 1999 (79)	Preterm
Agostoni et al, 1999 (80)	Only DHA and AA reported
Henderson et al, 1998 (81)	Few FAs reported
Fidler et al, 1998 (82)	Pooled sample
Carnielli et al, 1998 (83)	Preterm
Clandinin et al, 1997 (84)	Preterm
Makrides et al, 1996 (85)	Pooled sample
Jacobs et al, 1996 (86)	Preterm
Foreman-van Drongelen et al, 1996 (87)	Preterm
Beijers and Schaafsma, 1996 (88)	Preterm
Ruan et al, 1995 (89)	Packed column
Luukainen et al, 1995 (90)	Banked samples
Glew et al, 1995 (91)	Packed column
Jackson et al, 1994 (92)	Packed column
Hoffman et al, 1993 (93)	Preterm
Spear et al, 1992 (94)	One subject only
Sanders et al, 1992 (6)	Packed column
Dotson et al, 1992 (95)	n not provided
Prentice et al, 1989 (96)	Pooled sample
De-Lucchi et al, 1988 (97)	Packed column
Specker et al, 1987 (4)	Few FA reported
Kneebone et al, 1985 (98)	Packed column
Finley et al, 1995 (99)	Packed column
Harris et al, 1984 (100)	One subject consumed fish oil
Okolska et al, 1983 (101)	Packed column

(Continued)

Table 2.2 (Continued)

Reference	Reason for exclusion
Harzer et al, 1983 (102)	Pooled sample
Bitman et al, 1983 (103)	Packed column
Putnam et al, 1982 (104)	Packed column
Jansson et al, 1981 (105)	Packed column
Gibson and Kneebone, 1981 (106)	Packed column
Gibson and Kneebone, 1980 (107)	Analysis of colostrum
Hall et al, 1979 (10)	Packed column

1 TG, triacylglycerol; DHA, docosahexaenoic acid; AA, arachidonic acid; FA, fatty acid.

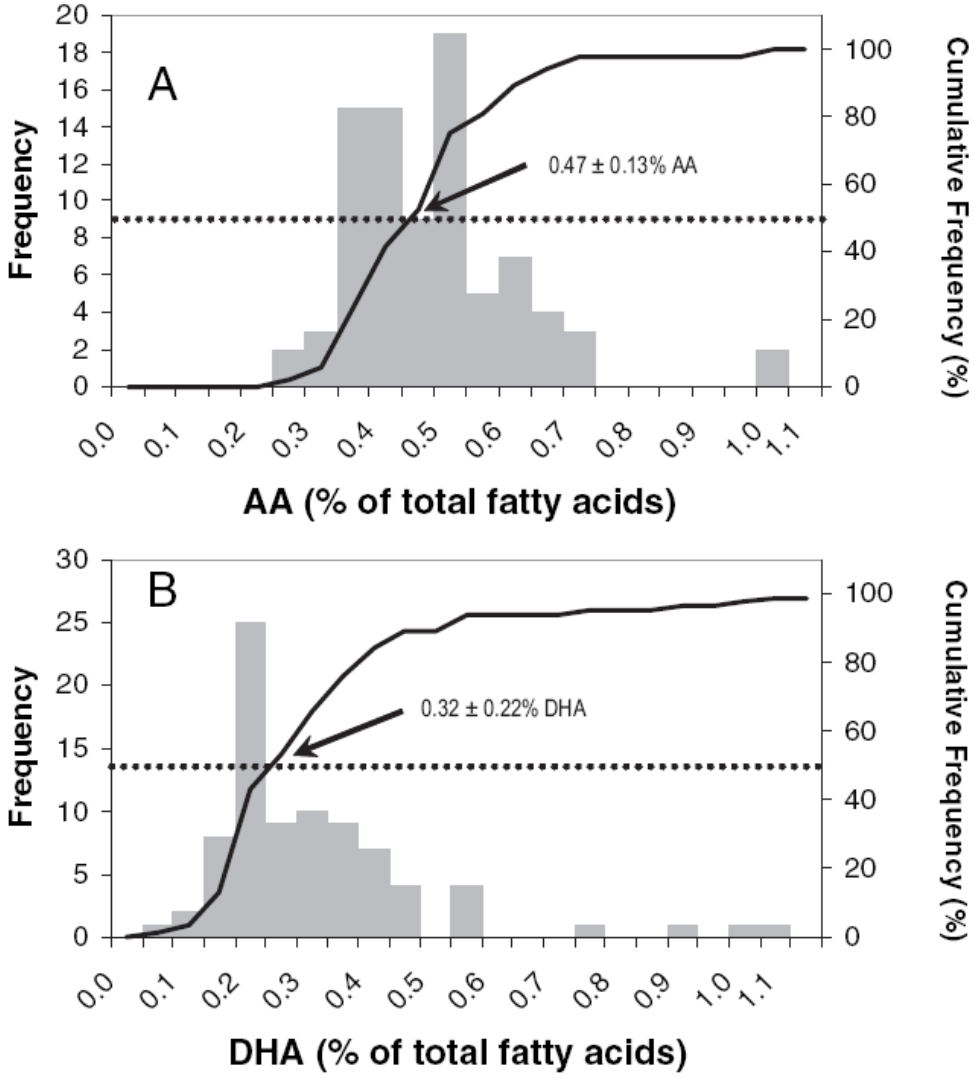

Figure 2.1. Distribution of arachidonic acid (AA) and docosahexaenoic acid (DHA) in the primary analysis. The arrow refers to the location of the average at the 50th percentile.

variability, each of which contributes variance to the overall spread in the data. It is not possible to reliably estimate the relative contributions of each of these 2 components of variability from so many studies. However, we note that the typical analytic test-retest precision for capillary GC analysis of FAs of 0.1–1.0% abundance is ~0.1%, and there is no reason to expect that the analytic variance for DHA should differ from that of AA. We can confidently assign excess variation in the data to real biological variability, induced primarily by diet but by other factors as well. We conclude that the excess variance in DHA distribution is evidence of the tighter control of AA concentrations in breast milk, which is consistent with many other data, which show that tissue AA concentrations are more refractory to dietary manipulation than are DHA concentrations(108). A plot of AA versus DHA concentrations for the primary analysis group is shown in Figure 2.2. The correlation was significant ($r = 0.25$, $P = 0.02$), which indicated that the prediction of the concentration of one mean LCPUFA from the other is nearly meaningless for a set of regional samples. This implies that the correlation of DHA and AA in any particular breast-milk sample is still lower because of the mathematical fact that the correlation between mean values is always greater than the correlation between data points making up those means. The shallow slope (0.15) shows that AA concentrations, on average, vary much less than do DHA concentrations, and inspection of the plot indicates that the significance of the slope is driven by a few high values for DHA.

Using strict selection criteria for data quality in this meta-analysis, we found that worldwide mean DHA and AA concentrations in human milk are 0.32 ± 0.22% and 0.47 ± 0.13%, respectively.

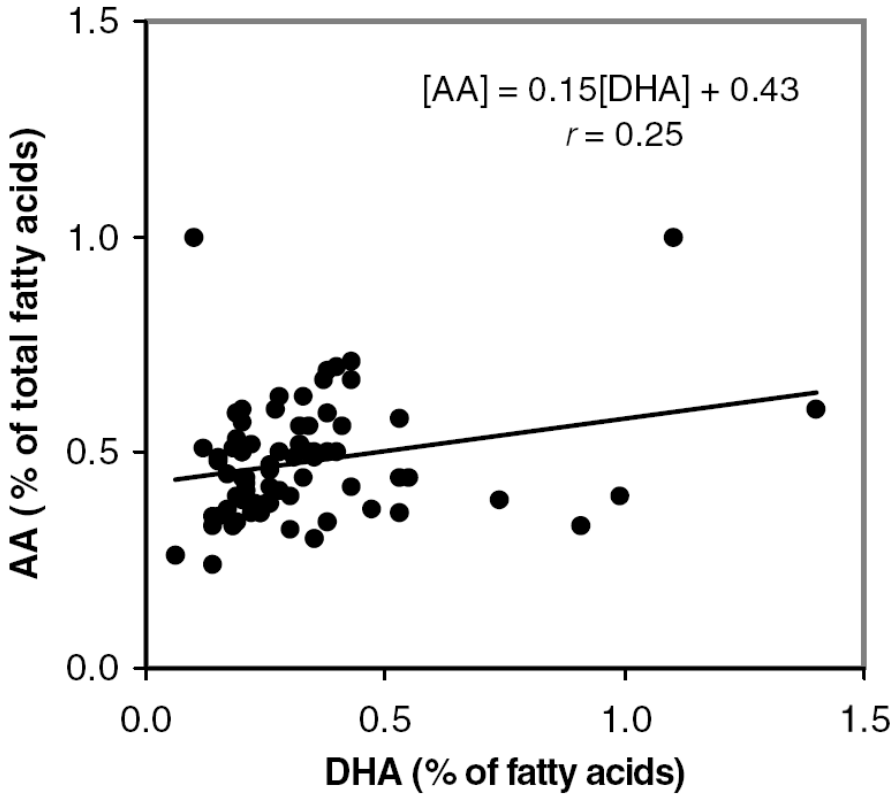

Figure 2.2. Mean concentrations of arachidonic acid (AA) versus docosahexaenoic acid (DHA) in breast milk. The slope is significant (P = 0.02).

2.4 Discussion

There are ≥2 ways to compute worldwide mean LCPUFA values, both of which have inherent weightings that should be borne in mind. A simple mean of mean values, as we computed, is inherently weighted evenly by study and against the number of subjects in each study. For instance, a study with 8 subjects is weighted the same as a study with 100 subjects. It is also biased toward regions in which more studies have been conducted, and away from regions in which fewer have been studied. This procedure has the advantage of effectively estimating a mean for each study population, which then contributes one data point (for DHA) to the meta-analysis. An alternative is to compute mean DHA and AA values by using weightings according to the number of subjects in each study. This mean is biased toward studies, and therefore regions, in which most of the subjects have been enrolled, and intuitively we see no rationale for doing so. Nevertheless, we computed this mean for comparison with our reported value. The weighted mean DHA was 0.32%, equivalent to the non-weighted mean, and thus the 2 approaches yield the same result. The AA weighted mean was 0.45%, which represents a deviation of –0.02% from our reported value. There are data from many more natives of developed countries than for natives of traditional cultures, and this selection bias may have contributed to the deviation. Nevertheless, the magnitude of the deviation is a fraction of the AA SD, 0.13%. We know of no data to suggest that a difference of this magnitude is biologically significant.

Concentrations of DHA and AA in breast milk depend on the amount of these preformed FAs in the mother's diet and their biosynthesis from precursors.

Milk DHA content appears to be closely linked to maternal dietary DHA intake, with dose-dependent linear increases in breast-milk concentrations of this nutrient with increased maternal intake (85).

In our study, the 5 locales with the greatest breast milk DHA concentration are Canadian Arctic, Japan, Dominican Republic, Philippines, and Congo (1.4–0.6%); all but Congo are coastal or island populations that have a high marine food intake. In contrast, the lowest breast-milk DHA values are for Pakistan, rural South Africa, Canada, the Netherlands, and France (0.06–0.14%). These populations are either inland or are developed countries, both of which are usually associated with low marine food consumption. Thus, the extreme values are consistent with studies suggesting that marine food–consuming populations have greater breast milk DHA concentrations(7, 8). The response of milk AA concentrations to maternal dietary AA intake is less predictable than that of DHA and may be more sensitive to the profile of other maternal dietary FAs(30).

Several studies have shown that the biosynthesis of DHA and AA from precursors is low: in 2 studies of men, <0.01% of labeled linolenic acid (18:3n-3) was converted to DHA as measured in plasma(109, 110), although there is evidence that conversion is greater in women(111). Importantly, sustained high supplementary dietary linolenic acid (10.7 g/d) did not increase breast-milk DHA(20). The majority of AA in milk was not from dietary LA conversion but rather from maternal stores(112). The weight of current evidence is that biosynthesis of DHA and AA is low, and augmentation of breast-milk DHA and possibly AA during lactation is best accomplished by consumption of

preformed DHA and AA. The higher variability of DHA than of AA is consistent with the conclusions of a recent study, which was included in the present analysis(11).

This study conducted a comprehensive analysis of FA profiles in breast milk from women from 9 countries and concluded that DHA was the most variable of all the FAs, and that AA was much less so. The best estimates of worldwide mean breast-milk DHA and AA concentrations (wt:wt) from the primary analysis group are 0.32 ± 0.22% for DHA and 0.47 ± 0.13% for AA. These means are not much different from those obtained by weighting according to numbers of subjects and are lower than those obtained in studies that used packed columns and protocols that fall outside the other inclusion criteria. The correlation between DHA and AA is surprisingly low, which reflects a high degree of variability in the ratio of DHA to AA in individual breast-milk samples. In summary, this review of the literature describes worldwide breast milk DHA and ARA concentrations using strict inclusion criterion and can be used as a guide to infant feeding.

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CHAPTER 3

POSITIVE IDENTIFICATION AND QUANTIFICATON OF *TRANS* MONOENE FATTY ACIDS IN HUMAN CEREBELLUM AND PARIETAL LOBE

3.1 Introduction

While the specific metabolic role and physiological function of long-chain polyunsaturated fatty acids has been demonstrated in a number of broad *in* vitro and *in* vivo studies, much less is known about the precise physiological role of trans fatty acids in mammalian systems and specifically the central nervous system. Trans fatty acids (TFA) are unsaturates that contain at least one double bond in the *trans* (*E*) configuration. Many positional isomers are possible since the double bond can be located anywhere along the hydrocarbon chain. TFA enter the food supply as byproducts of chemical hydrogenation intended to increase shelf life and alter the physico-chemical properties of oils, notably melting point, to enhance the palatability of foods(1). Specific *trans* isomers also occur naturally as components of ruminant fats generated by bacterial biohydrogenation(2).

The major food-based *trans* isomers are the C18 *trans*-monoene FA found in industrial partially hydrogenated vegetable oils (PVHO) such as 18:1n-9, elaidic acid, and biohydrogenated ruminant fats (18:1n-7, *trans*-vaccenic acid) (3, 4). These TFA have double bonds generally located between the C6 and C12 carbons. TFA are also intermediates of normal fatty acid metabolism, however these TFA are found at trace concentrations in tissues and are

structurally different than dietary TFA in that the *trans* double bond is located adjacent to the carboxyl group.

The distribution of dietary TFA in mammalian tissue has been explored since the 1970s(1). For instance, TFA have been identified in plasma, erythrocytes, liver, kidney, testes, heart, adrenals, adipose, and ovaries from 2.4 – 11.5% in rats fed *trans*-18-1 at 12% of FA over a four month period(5). Tissue concentrations are dose-dependent and vary among different lipid classes (cholesterol esters, triacylglycerols, phospholipids). The brain is notably absent from the list because the few scattered reports of TFA in the central nervous system (CNS) have not used methods with sufficient specificity to unambiguously identify TFA at the levels at which they may exist. Conventional, high performance methods for detection of TFA, specifically high resolution gas chromatography and tandem mass spectrometry with collisionally activated dissociation, are not sufficiently selective to isolate and identify TFA isomers at low concentrations from the high concentration of *cis*-monoenes in most natural fats.

Dietary TFA are of intense public interest because of relatively recent associations with negative or positive health effects. Industrially produced PVHO have come under scrutiny with recent epidemiological evidence that their intake is associated with an increased risk of coronary events(6). Population-based studies have demonstrated that PVHO intake leads to a high LDL, low HDL cholesterol profile(7) and has positive associations with cardiovascular disease and diabetes(8, 9). While at least one study has suggested that dietary TFA intake is associated with diseases of the brain

such as Alzheimer's disease (10), there are as yet no reports of TFA in the CNS, and no studies have been performed on the potential physiological role of TFA in nervous tissue. On the other hand, specific TFA of ruminant fats show potent anticarcinogenic activity(11), specifically *cis*-9*, trans*-11-18:2, which is biosynthesized from *trans*-11-18:1 by stearoyl CoA desaturase (12).

We applied a highly sensitive method to detect TFA in parietal lobe and cerebellum autopsy specimens of normal aged (NA) and histologically confirmed Alzheimer's disease patients. Analysis was by gas chromatography-covalent adduct chemical ionization tandem mass spectrometry (CACI-MS/MS)(13-18), previously shown to be highly selective and quantitative for monoene isomers in the presence of much higher concentrations of the more abundant *cis* monoenes (19).

3.2 Methods

Sample preparation. Five male and five female subjects who died at age 61.5 ± 6.9 years (mean ± SD) donated parietal lobe and cerebellum in the context of a rapid autopsy protocol (20). For AD subjects, duration of disease ranged from 1-9 years. Samples were maintained at –80^{o}C until processing. Since most samples were stored at –80^{o}C for 4+ years, RNA was extracted and evaluated for integrity using a ratio of absorbencies at 260 nm and 280 nm. RNA are among the most labile biomolecules because of rapid digestion by ubiquitous RNases; their preservation is indicative of good postmortem tissue preservation (21-23). RNA was isolated using the RNeasy® RNA isolation kit (Qiagen, Valencia, CA, USA). The A260/A280 ratios ranged from 1.79 to 1.95

(1.88 ± 0.06, mean ± SD), where a ratio 1.8 or greater is considered an acceptable indicator of RNA preservation (24).

Tissues were thawed on ice and aliquoted (~100 mg) into screw capped tubes. All reagents were of analytical grade and mixtures were made fresh before use. 1,2-Diheptadecanoyl-sn-glycero-3-phophatidylcholine (Matreya, Inc. State College, PA USA) was added to each sample as an internal standard. The internal standard is a 17:0 phospholipid which closely resembles the lipids to be analyzed in its chromatographic properties, but does not occur naturally in mammalian tissues. It is added when the tissue is first extracted so it is carried through the extraction, separation and methylation, as well as through chromatography. The area of all the sample peaks are then related to that of the internal standard, the absolute amount of which is known. In our laboratory, GC is used for routine analysis of lipids. Thus, we have set up and regularly test our system for quality control with known standards in order to ensure that the equipment is correctly functioning, and that it is not subject to gradual deterioration or random variation.

A modified single extraction/derivatization method was used to prepare total fatty acid methyl esters for analysis (25) as described in detail in the appendix. FA extraction and transesterification was performed by addition of an aqueous and organic mixture to the tissue. The aqueous reagent mixture consisted of methanol, 2, 2-dimethoxypropane, and concentrated sulfuric acid 85:11:4 by volume; 1.4 ml total was added to each sample. The organic reagent mixture contained heptane and toluene 63:37 by volume; 1.6 ml total was added to each sample. Heptane was added to bring the total volume of each tube up to

5 ml. All samples were subsequently incubated at 85°C in a shaking water bath for 120 minutes. After incubation, 2ml of saturated NaCl was added to assist the separation of the organic and aqueous layers. After additional heating, the heptane layer containing the fatty acid methyl esters (FAME) was collected and dried under N_2.

Instrumentation. All GC-MS/MS analyses were performed with a Varian Star 3400CX gas chromatograph operated in splitless mode, coupled to a Varian Saturn 2000 ion trap tandem mass spectrometer (Varian Inc., Walnut Creek, CA, USA). A BPX70 capillary column (60m×0.32 mm×0.25µm; SGE Inc., Austin TX, USA) was used for all analyses. The column temperature and injector parameters for both CIMS and CIMS/MS analysis were as follows: Injector temperature was maintained at 250°C in splitless mode with a purge at 0.85 min after injection, initial column temperature was 80°C ramped up to 200°C at 50°C/min and held for 5 min then ramped to 220°C at 4°C/min for 12 min, total run time 24.4 min. Optimal [M+54] formation was obtained by adjusting the CI gas inlet valve to obtain an *m/z* 42 (MH) to 54 (MIE) ratio of about 6 with the acetonitrile reservoir at ambient temperature. These methods have been described in detail elsewhere (26).

FAME Quantification. *Trans* 16:1n-7 and 18:1n-9 FAME standards were obtained from Matreya, Inc. (State College, PA USA). All peak areas were derived by plotting the MS-2 diagnostic ions and using areas under the curve calculated with Varian Saturn software (version 5.1). A calibration curve was produced by running four different concentrations of the standards in triplicate in the linear range of peaks of interest. TFA concentrations below 1.0 ng

FAME / mg brain tissue were judged to be below quantifiable limits and are listed as “trace”. Differences between and within brain regions by fatty acid isomer and disease state were tested using Students t-test in Microsoft Excel and ANOVA in JMP 5.1 (SAS Institute, Cary, NC).

3.3 Results

Figures 3.1A and 3.1B, respectively, present parietal lobe and cerebellar total brain FA concentrations (means + SD). There were no significant differences found between major fatty acid concentrations in NA or AD subjects ($p>0.05$). Targeted analyses by CACI-MS/MS revealed a series of ten monoene FAME 16 or 18 carbons in length. Diagnostic ions of peaks with retention times revealing *trans* double bonds were used to generate plots indicative of double-bond positions in 16 and 18 carbon monoenes as described previously (26).

Table 3.1 shows profiles of monoenic TFA acids 16 and 18 carbons in length in parietal lobe and cerebellum, expressed as a percent of total TFA. In cases for which a specific TFA were below detection limits for some but not all subjects, we report a mean and SD for those subjects for which the TFA were detectable only.

The predominant TFA are *trans*-18:1n-9 and *trans*-18:1n-7 constituting almost half and a quarter, respectively, of all TFA detected in all samples. In NA parietal lobe, none of the minor 18:1 TFA were detected in any samples, though they were detected in most but not all of the NA cerebellum, where *trans*-18:1n-8 was averaged about 10% of TFA in four of five specimens.

Similar results were obtained for the AD samples, though *trans*-18:1n-8 and *trans*-18:1n-6 were observed in a few samples of parietal lobe and cerebellum. Of TFA for which all samples were above detectable concentrations, there were no significant differences in TFA acid concentrations found between NA and AD subjects in either brain region analyzed ($p>0.05$). Unlike the *trans*-18:1, no *trans*-16:1 isomer predominates in any group. All *trans*-16:1 were detected in all parietal lobe samples at relative mean concentrations of 3.8 to 7.5%, except for 16:1n-8 which was not detected. Results for the cerebellum were similar, though here the *trans*-16:1n-8 isomer was detected in half the samples, and the *trans*-16:1n-6 isomer was not detected in four of ten samples.

Table 3.1 also reports the total TFA detected in each of the groups. Means appear in a relatively small range, from 122 to 160 μg/mg tissue.

3.4 Discussion

We report, for the first time, positive identification of ten monoenic TFA in the postmortem brains of normal and AD human subjects, specifically parietal lobe and cerebellum. The quantitative results in Table 3.1 provide clues to the origin of TFA in the human CNS. The most prominent TFA in all samples is *trans*-18:1n-9(elaidic acid). Elaidic acid is generally a major *trans* monoene in partially hydrogenated vegetable oils because it results from direct isomerization of oleic acid, the most prominent *cis* monoene in food (27). This process, however, usually results in an envelope of intensities of *cis* and *trans*-18:1 isomers with double bonds from positions 6 to 16. In contrast, the major

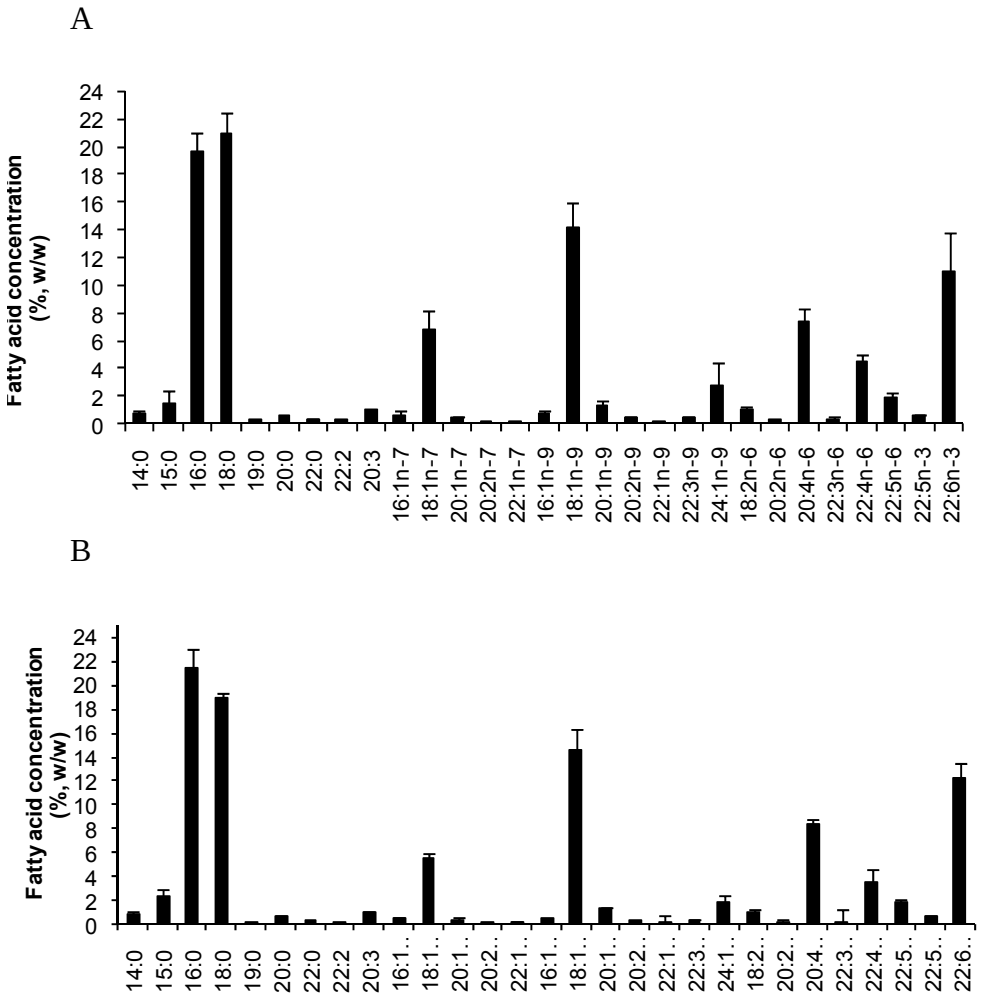

Figure 3.1. Pooled parietal lobe (A) and pooled cerebellum (B) fatty acid profiles of control and AD subjects (FA expressed as wt% total).

Table 3.1. *Trans* fatty acid profiles (mean ± SD, %w/w of *trans* FA) and concentrations (ng FAME/mg tissue) in parietal lobe and cerebellum autopsy specimens from normal aged (NA) and Alzheimer's disease (AD) human subjects.

	Parietal lobe		Cerebellum	
Fatty acid	NA	AD	NA	AD
trans-16:1n-10 (*trans* 6-16:1)	5.2 ± 2.6	5.1 ± 2.0	6.7 ± 3.3	6.1 ± 2.8
trans-16:1n-9 (*trans* 7-16:1)	6.2 ± 3.9	5.3 ± 2.5	5.9 ± 3.0	7.0 ± 4.0
trans-16:1n-8 (*trans* 8-16:1)	tr*	tr	3.7 ± 5.1 (2†)	3.5 ± 3.4 (3)
trans-16:1n-7 (*trans* 9-16:1)	7.5 ± 5.2	6.0 ± 2.6	10 ± 5.5	8.6 ± 3.4
trans-16:1n-6 (*trans* 10-16:1)	4.3 ± 2.4	3.8 ± 1.4	2.2 ± 2.1 (3)	2.0 ± 1.9 (3)
trans-18:1n-10 (*trans*-8-18:1)	tr	tr	0.9 ± 1.9 (1)	1.2 ± 2.5 (1)
trans-18:1n-9 (*trans*-9-18:1)	53 ± 30	43 ± 17	37 ± 17	43 ± 15
trans-18:1n-8 (*trans*-10-18:1)	tr	10 ± 13 (3)	11 ± 8 (4)	8.3 ± 7.6 (3)
trans-18:1n-7 (*trans*-11-18:1)	24 ± 14	22 ± 13	19 ± 10	19 ± 10
trans-18:1n-6 (*trans*-12-18:1)	tr	4.3 ± 7.3 (2)	3.4 ± 3.6 (3)	2.1 ± 3.0 (2)
total *trans* (ng FAME/mg tissue)	156	140	160	122

* trace, below quantifiable limits (<1.0 ng/FAME) for all subjects
† number of subjects for which means were quantifiable in that group (each group n=5); mean and SD are for those subjects within quantifiable limits of *trans.* No statistically significant differences exist between NA and AD groups for any *trans* isomers detected.

trans monoenes of dairy fat is 18:1n-7 (*trans*-11-18:1), with 18:1n-9 (*trans*-9-18:1) being the second most prominent. NA parietal lobe shows strong signal from 18:1n-7 and 18:1n-9 with other isomers below quantifiable limits. While these TFA are also at highest concentration in AD parietal lobe, 18:1n-8 and 18:1n-6 are also at substantial concentrations.

Figure 3.2 compares our pooled data on brain TFA with estimated consumption of TFA in North American foods from 1996-1999, adapted from Wolff et al. (27). *trans*-18:1n-9 is the most prominent brain TFA, and is also the most prominent dietary TFA, coming primarily from PHVO. Similarly, *trans*-18:1n-7 is the second most abundant TFA in brain and is also a major component of dietary *trans*, but about half originates from PHVO and half from ruminant fat. *trans*-18:1n-8 is notably lower in ourbrain samples than from food sources. The figure demonstrates that that dietary intakes of TFA isomers from North American foods are qualitatively similar to those obtained from our samples.

An older report from 1978 shows that the rat liver phospholipid *trans*-18:1n-8 is selectively depleted relative to *trans*-18:1n-9 and *trans*-18:1n-7 compared to the dietary *trans* distribution (28). The liver triacylglycerol TFA distribution is nearly indistinguishable from the dietary input. Liver is a major source of fatty acids for the brain. Brain lipids are predominantly found as phospholipids and have very low concentrations of triacylglycerol, thus the TFA distribution in brain is consistent with selectivity against the *trans*-18:1n-8 isomer in PL, possibly originating in the liver, a prominent source of brain fatty acids. Finally, PHVO and ruminant fat contains about 20% *trans* isomers with the

double bond at position 4-7, and 13-16, none of which were detected in any of the brain samples.

In situ *cis*-*trans* isomerization is another possible origin of *trans*-18:1. Figure 3.1 shows that oleic acid (*cis*-18:1n-9) and vaccenic acid (*cis*-18:1n-7) are the two most abundant *cis* monoenes. The intensity ratio is similar to that found for the corresponding *trans* isomers, a condition that would be expected if non-specific isomerization were operating. Thermal, non-catalyzed *cis*-*trans* isomerization requires high energy, similar to that required for bond breaking, as might be available if samples were heated. Other changes would also be expected, including rapid degradation of brain RNA, which was not observed. On the other hand, active metals or other catalysts, as might be released as cells and organelles die, could locally release active species capable of catalyzing isomerization, and this possibility cannot be ruled out by our data.

trans-16:1 are normally of very low concentration in PHVO because the parent *cis*-16:1 is of very low concentration (29), however isomers with double bond positions from 4 to 14 are found in most ruminant fats, with the predominant *trans*-16:1n-9 present at 5-10 fold greater abundance than the n-8 and n-10 isomers (30). This strongly implies a ruminant fat origin for the *trans*-16:1 isomers. The brain distribution does not dramatically favor any particular isomer, though *trans*-16:1n-7, the predominant dietary 16:1 TFA in ruminant fats, is numerically greater in all groups. Similar to *trans*-18:1n-8, *trans*-16:1n-8 is at notably low levels; it is at trace concentration in parietal lobe and is quantifiable in only 5 of 10 cerebellum samples.

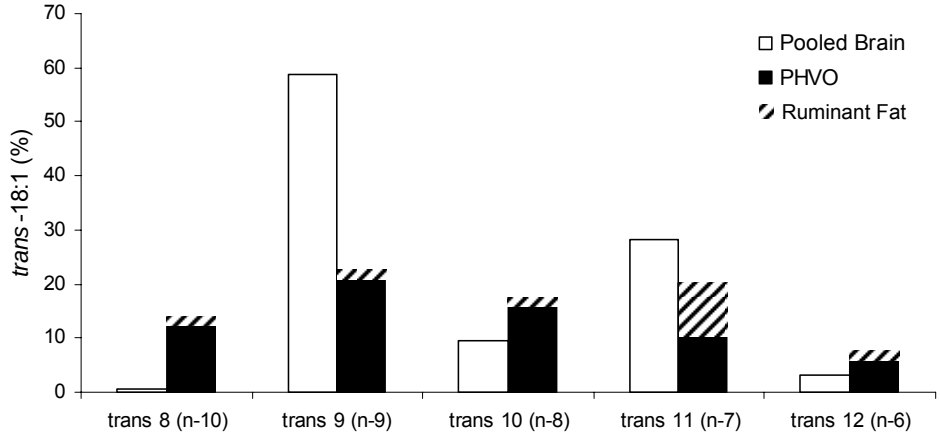

Figure 3.2. Pooled brain fatty acid profiles (wt% total) presented alongside estimated profiles of North American *trans*-18:1 intakes in the late 1990s; after Wolff et al. (27).

We know of no data to indicate whether there is any selection against *trans*-16:1n-8, similar to the *trans*-18:1n-8 data, in PL synthesis. Chain shortening in the CNS has been previously demonstrated in rodents (31), and thus this mechanism may operate to generate *trans*-16:1n-8 from *trans*-18:1n-8, or indeed any of the *trans*-16:1 isomers.

The concentrations and pattern of TFA distribution was similar for NA and AD specimens, though there were minor differences in the abundance of specific *trans*-18:1. Notably, *trans*-18:1n-8 was at trace levels in parietal lobe for NA specimens but easily detected for three of five AD specimens. *trans*-18:1n-6 was also present in two of five AD specimens and not detectable in NA, and these corresponded to subjects for which *trans*-18:1n-8 was found (data not shown). Neither of these associations support a strong connection between TFA and AD, though we may hypothesize that the unknown mechanisms that select against *trans*-18:1n-8 in liver are related to the changes seen in AD. There were no obvious differences between NA and AD in cerebellum.

The specific metabolic consequences of the presence of TFA in the brain remains unclear. While one epidemiological study indicates a relationship between *trans* intake and risk of neurodegeneration (32), metabolic studies of the role of TFA in the CNS are limited. Though TFA have been shown to decrease concentrations of neuroprotective DHA in plasma and liver (33), future work is necessary to establish any clinical relevance of such an association.

Positive identification of TFA in the CNS raises many research questions,

specifically the transport, metabolism and physiological roles of these fatty acids in nervous tissue. While region specific differences exist in other lipids measured in the brain, we report no statistically significant differences between parietal lobe and cerebellum for *trans* fatty acids. The effects of PHVO-derived TFA have been studied in a number of other tissue types and disease states.

Most notably, a large body of population-based evidence indicates that TFA consumption is associated with an increased risk of coronary disease (8, 34-39). Mechanistically, TFA are implicated to have an indirect effect on cholesterol ester transfer protein activity in the hepatocyte leading to increased HDL clearance (40-42). TFA are hypothesized to interact with receptors in endothelial cells, increasing NF-κB expression and endothelial dysfunction by up regulating E-selectin and other cell adhesion molecules (43, 44). In adipocytes, TFA increase free fatty acid levels and decrease adipocyte insulin sensitivity (45). Finally, TFA may interact with monocytes or macrophages leading to increased inflammatory response via TNF-α, IL-6, and C-reactive protein (43, 46). In contrast to these negative health effects, the major ruminant-derived TFA, *trans*-11-18;1 (*trans*-vaccenic acid) is a precursor to the conjugated linoleic acid *cis*-9, *trans*-11-18:2. This diene TFA has potent anticarcinogenic activity in rats (12). Importantly, however, we found no evidence in any brain samples of the presence of these *trans* or conjugated dienes.

Because of a general associations between TFA and increased disease risk, Larque et al. (47) suggest that the brain possesses a protective mechanism to

limit the transport of monoenic TFA into the CNS. Studies that rely on infusion of isotopically-labeled nonesterified FA bound to albumin in brain perfusion (48) or whole animals (49, 50) find facile transport of SFA and monenes into the CNS. In contrast, studies that infuse labeled fatty acids into the stomach of neonatal rats show transport of SFA into liver, lung and other organs but not into the brain; however polyunsaturated fatty acids are transported into the brain (51, 52). We are not aware of any studies of TFA transport into the brain. Our results indicate such studies are necessary to establish whether there is selectivity depending on double bond position and/or geometry for transport of fatty acids across the blood-brain barrier, and if so, why such a protective mechanism may exist. Studies of bioactivities of specific TFA in nervous tissue are also warranted. In summary, the quantitative distributions of these *trans* fatty acids are consistent with their origin from diets that are a composite of dairy and partially hydrogenated vegetable oil *trans* sources, and describe the presence of these lipids in the human brain for the first time.

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CHAPTER 4

DIETARY RESVERATROL INDUCES PROTECTIVE CHANGES IN PROTEIN LEVELS OF TRANSTHYRETIN, DREBRIN, AND GLYCOGEN SYNTHASE KINASE 3-BETA IN MICE

4.1 Introduction

As humans age, cognitive setbacks occur regardless of genetics and diet. Dementia is a general term that describes any syndromes characterized by multiple cognitive deficits that lead to impairments in occupational and social functioning. Dementia is largely broken down into two discrete classes of illness: Alzheimer's Disease and vascular dementia(1).

Alzheimer's disease (AD) is a progressive, age-dependent neurodegenerative disorder resulting in cognitive impairment of the brain that is specifically characterized by losses in short-term memory and plaque deposits in the brain. While the decline observed during AD involves multiple factors that influence several systems, the specific pathogenesis of the disease is still poorly understood. It is widely hypothesized that increases in amyloid beta protein, a product of sequential proteolysis of amyloid precursor protein, leads to neurotoxic amyloid beta 1-42 aggregates, causing downstream oxidative damage, neuroinflammation, and hyperphosphorylation of microtubule associated tau-proteins resulting in neurofibrillary tangles and neuronal death. As such, the presence of intraneuronal amyloid beta (Aβ) plaques and neurofibrillary tangles in the cortex and hippocampus with concomitant neuronal and memory loss are the hallmarks of AD(2). Although AD's precise

cause is unknown, a number of risk factors are involved in AD onset such as age(3), ApoE4 genotype(4), and diet(5). Despite several FDA approved drugs demonstrating moderate symptomatic benefits, no available treatments have been shown to stop the progressive loss of cognitive function manifest in AD(6).

Animal and epidemiological studies support that polyphenol constituents of red wine possess bioactivities that may afford protection against cardiovascular disease and possibly, central nervous system disorders such as Parkinson's, Huntington's, and Alzheimer's disease(7). To date, a number of studies have examined dietary factors and neurodegeneration, and several naturally-occuring plant compounds have been tested in treating AD(8). One of the most promising compounds to emerge has been resveratrol, a naturally occurring polyphenol in grape skin and red wine(9).

A number of *in vitro* studies have demonstrated resveratrol's ability to protect against neuronal degradation(10) and reduce levels of secreted and intracellular amyloid beta peptides(11). It is well established that a major feature of resveratrol's neuroprotective activity is due to its action as a calorie restriction mimetic(12, 13), thereby inducing the sirtuin family of proteins whose upregulation is associated with neuroprotection in several AD models(14-16). *In vivo,* AD transgenic mice consuming a Cavernet Sauvignon red wine for 7 months demonstrated improved spatial-memory functions and decreased Aβ peptides(17). Further, resveratrol reduced neurodegeneration and cognitive decline in mice expressing a coactivator of cyclin-dependent kinase 5 and displaying massive forebrain degeneration with AD features(18).

In another study, resveratrol was shown to reduce plaque pathology in an AD transgenic mouse model(19).

While a number of mechanisms for resveratrol's protective effects in AD have been proposed, further identification and elucidation of targets are needed. Several studies suggest that resveratrol may also act on a number of sirtuin-independent targets that lead to neuroprotection(20, 21). *In vitro*, glycogen synthase kinase 3, a protein central to a variety of biological processes including neurodegeneration, and transthyretin, a Aβ scavenger, are modulated by resveratrol (22, 23). Further, resveratrol's ability to modulate postsynaptic events suggest its neuroprotective benefits also be exerted at the synapse(24, 25). Drebrin is a key postsynaptic protein critical to maintaining synaptic function, losses of which have been reported in AD(17). We report here a test of the hypothesis that resveratrol modulates these proteins *in vivo* in AD transgenic and wild-type mice. The objective of this study was to examine whether dietary resveratrol altered the levels of a number of specific protein targets specific to AD and neurodegneeration.

4.2 Materials and Methods

Animals. 41-44 week old B6.Cg-Tg(APPswe,PSEN1ΔE9)85Dbo/J were purchased from Jackson Laboratories (Place, Maine). These transgenic mice express two mutations associated with early-onset AD; a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9). These mice normally develop Aβ plaque at six to seven months of age with progressive increases in plaque up to 12

months(26). The mice used in the study were singly housed in individually vented cages at Cornell University's Biotechnology Mouse Facility at a constant temperature (71 ± 1°F), humidity (44 ± 4%) and illumination (12 h light/dark cycles) with food and water provided ad libitum. All procedures with the animals were approved by Cornell University's Institutional Animal Care and Use Committee.

Treatment. A total of 18 male mice were used, 9 wild-type and 9 transgenic. A 9 week acclimation period occurred whereby all mice were introduced to singly housed cages at Cornell's facility and fed AIN-93G diet *ad libitum,* and dietary regimens began at 50-53 weeks of age*.* Of the wild-type mice, 6 mice were assigned to receive control diet group and received a standard AIN-93D diet (Dyets Inc, Bethlehem, PA) while 3 mice were assigned to receive resveratrol at 0.19% w/w mixed homogenously into AIN-93G. Of the transgenic mice, 3 mice received the control diet and 6 mice received the resveratrol-supplemented diet (same formulations as above). The daily dosage in mice is 174 mg/kg/d (3.3 g food per day for a 36 g mouse). The equivalent dose in humans is 14 mg/kg or 0.98 g per day for a 70 kg individual. The dietary regimen lasted 16 weeks. Resveratrol (>98%) was purchased from Orchid Pharmaceuticals (Aurangabad, India) and mixed to homogeneity in the dark during manufacturing of the diets. Diets were stored at 4°C and replaced in all cages weekly. All animals were inspected daily while body weight and food intake measurements were performed on a weekly basis throughout the experimental period.

Tissue preparation. After 16 weeks of the dietary intervention, mice were

sacrificed by CO_2 inhalation and rapidly dissected. Brains were removed and a thin coronal section containing cortex and hippocampus was excised using a rodent brain matrix and fixed in 10% neutral buffered formalin. The rest of the brain was separated into several regions, flash frozen in liquid nitrogen, and stored at -80°C until analysis.

Immunoblot analysis. Cortex was homogenized in ice-cold lysis buffer (150mM NaCl, 1% Triton X-100, 1mM EDTA, 50mM Tris pH 7.5) with protease (Protease inhibitor cocktail, Sigma Aldrich, St. Louis, MO) and phosphatase (PhosSTOP Roche, Indianapolis, IN) inhibitors as indicated by manufacturer. Samples were centrifuged for 4 min at 4^{o}C at 13,000 × g to obtain the soluble protein fraction. Protein concentrations were determined by a bicinchoninic acid (BCA) assay (Pierce Chemical Company, Rockford, IL). 25 µg of protein was loaded and electrophoresed by one-dimensional SDS-PAGE (12% w/v acrylamide), then electroblotted overnight onto 0.45 µm Immobilin-P PVDF membranes (Millipore, Medford, MA) and immunoblotted for drebrin (1:1000, Abcam, Cambridge, MA), insulin degrading enzyme (1:500, Abcam, Cambridge, MA), transthyretin (1:5000, Abcam, Cambridge, MA), total glycogen synthase kinase 3β (1:1000, Cell Signaling Technology, Danvers, MA), and phospho-glycogen synthase kindase-3β (Ser9) (1:1000, Cell Signaling Technology, Danvers, MA). Visualization of bands was accomplished using horseradish peroxidase-coupled (HRP) secondary antibodies and chemiluminescent substrates (West Dura, Pierce) with exposure to autoradiography film. Film images were digitized and analyzed using NIH ImageJ 1.63 software. Band intensities were normalized against corresponding bands for β-actin loading and transfer controls.

Immunohistochemistry. Coronal sections 5 μm thick were cut with a sliding microtome and processed as free floating sections at Cornell University's Histology Laboratory. Briefly, sections were washed with TBS pH 7.6 and incubated in 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. Sections were blocked using rabbit serum and incubated with monoclonal mouse anti-human beta-amyloid 6F/3D primary antibody, 1:50 (DakoCytomation, Glostrup, Denmark) in antibody diluent for 90 minutes. The secondary antibody, a biotinylated goat anti-mouse, was applied and the slides and incubated for 10 to 20 minutes at room temperature. Sections were then incubated in streptavidin-peroxidase conjugate for 10 minutes at room temperature. The chromogen, 3,3-diaminobenzidine-tetra hydrochloride (DAB from Dakocytomation) was applied to the slides for 1 minute at room temp and slides were counterstained using hematoxylin for 2 minutes and rinsed in distilled water. Slides were dehydrated using ethyl alcohol and cleared with xylene before being coverslipped using Permount mounting media (Fisher Scientific, Pittsburgh, PA).

Plaque counts and percentage occupied by the 6F/3D were quantified in the cortex and hippocampus. The region of interest was drawn manually under 4× magnification, the images were thresholded, and plaques were quantified using Metamorph 7.1 software (Molecular Devices, Sunnyvale, CA). The results of the analysis were confirmed in a blinded fashion by multiple researchers.

Brain Aβ ELISA analysis. Brain cortex was homogenized in carbonate buffer

(100mM Na_2CO_3, 50mM NaCl, pH 11) containing protease inhibitors (Protease inhibitor cocktail, Sigma Aldrich, St. Louis, MO). The homogenate was centrifuged at 14,000 x g for 20 min at 4°C. The supernatant (carbonate soluble) fraction was transferred to a new tube and stored at -80°C until analysis. The pellet was further homogenized in guanidine solution (5 M guanidine HCl in 50 mM Tris-HCl, pH 8.0). The homogenate was rocked for 4 hours at room temperature and centrifuged at 14,000 x g for 20 min at 4°C. After centrifugation, the supernatant (insoluble fraction) was transferred to a new tube and stored at -80°C until analysis. Soluble and insoluble Aβ40 and Aβ42 levels were determined using the Human β Amyloid 1-40 and 1-42 ELISA kits (Invitrogen, Camarillo, CA) according to manufacturer's protocol.

Statistics. Values reported are expressed as means ± SEM. $p < 0.05$ was considered significant. Statistical significance was tested by two tailed Students t-test and ANOVA using JMP 7 (SAS Institute Inc, Cary, NC).

4.3 Results

To determine the effects of resveratrol in both wild-type and AD transgenic mice on a number of key proteins involved in AD pathogenesis, western blot was used to measure levels of transthyretin, insulin degrading enzyme, and drebrin. Resveratrol significantly increased levels of transthyretin in mice consuming resveratrol compared to control diet (3.8-fold increase, Figure 4.1; $p < 0.05$, pooled data shown). However, resveratrol did not increase levels of TTR within transgenic mice, though the increase in TTR in the resveratrol-fed animals did approach significance ($p=0.07$). Resveratrol did not alter insulin

degrading enzyme levels in either wild-type or transgenic animals (Figure 4.2, pooled data shown). Resveratrol feeding significantly increased drebrin levels in both wild-type and transgenic animals (2.2-fold increase, Figure 4.2; $p < 0.05$, pooled data shown). To determine the effects of resveratrol on GSK-3 enzyme activity, total GSK3-beta and phospho-GSK3 β (ser9) were measured. Levels of phosphorylated GSK3-β were normalized to total levels of GSK-β. In both wild- type and transgenic groups, resveratrol significantly decreased GSK-3 β activity by increased phosphorylation at ser9 (Figure 4.3, phosphorylation at ser9; $p < 0.05$, pooled data).

To test the effect of dietary resveratrol on plaque pathology, transgenic AD mice were fed control (AIN-93G) or +Resv (AIN-93G with 0.19% resveratrol) diet for 16 weeks. Brain sections were stained with an antibody specific for extracellular beta-amyloid (Figure 4.4). Quantification of plaque areas revealed no significant differences in plaque burden in hippocampus or cortex between dietary groups. To determine the effect of dietary resveratrol on cerebral Aβ protein levels, Aβ contents in the carbonate soluble and insoluble (guanidine-soluble) fractions in the cortex were quantified by ELISA. No significant difference in soluble Aβ40 and Aβ42 was found between groups (Figure 4.5A). Further, no significant difference between insoluble Aβ40 and Aβ42 was found between groups (Figure 4.5B).

These results are consistent with the immunohistochemical analyses that showed no significant difference between Aβ plaque deposition in hippocampus and cortex between dietary groups.

Resveratrol did not alter body weight or food intake in APP/PS1 transgenic AD mice. Body weight and food intake measurements were recorded on a weekly basis throughout the experiment. Changes in body weight and total food intake did not vary between dietary groups (Table 3.1).

4.4 Discussion

In this study, aged AD transgenic mice fed dietary resveratrol for 15 weeks demonstrated increased protein levels of drebrin and transthyretin. Additionally, resveratrol-fed mice displayed increased phosphorylation of the protein glycogen synthase kinase-3 at serine 9 compared to controls. Resveratrol-fed mice did not demonstrate decreases in Aβ plaque load in hippocampus or cortex or secreted Aβ levels in cortex.

Glycogen synthase kinase 3, a serine/threonine protein kinase, was originally identified as an enzyme which regulates glycogen synthesis but is now known to affect a multitude of physiological events by interacting with a number of substrates(27, 28). Broadly speaking, GSK3 activity plays an important role in insulin resistance, tumorigenesis, inflammation, cardiac function, and neurodegeneration(28). Further, GSK-3 is intimately involved with memory formation, inflammation, as well as tau phosphorylation and other pathological hallmarks of AD, leading a number of researchers to classify it as a promising drug target and formulate the GSK3 hypothesis of AD(29-31). GSK activity is tightly regulated via its phosphorylation state, and its over-activation, which has been shown to occur in normally aged mammals(32), has been implicated

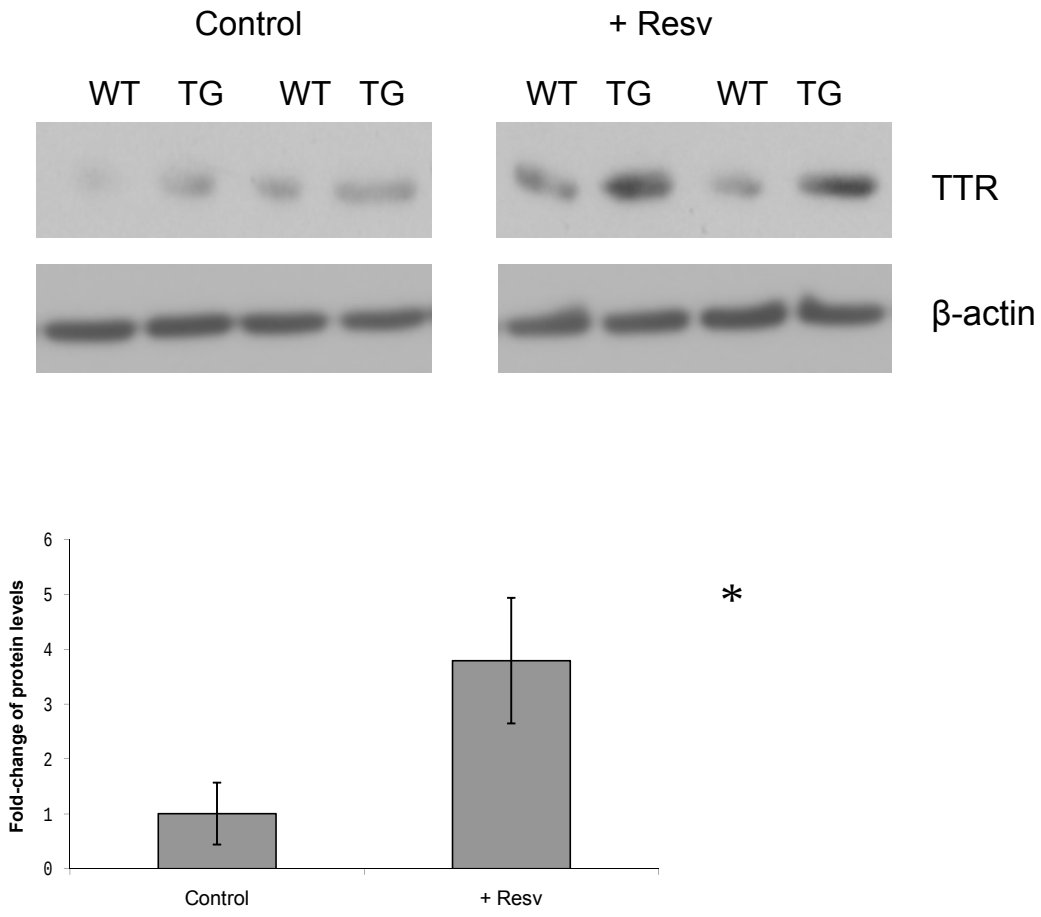

Figure 4.1. Resveratrol significantly increased levels of transthyretin in mice consuming resveratrol compared to control diet (3.8-fold increase; $p < 0.05$, pooled data shown). Resveratrol also increased levels of TTR within transgenic mice but not at statistically significant levels ($p=0.07$). Data represent means ± SEM of control (n=9) and resveratrol (n=9) groups.

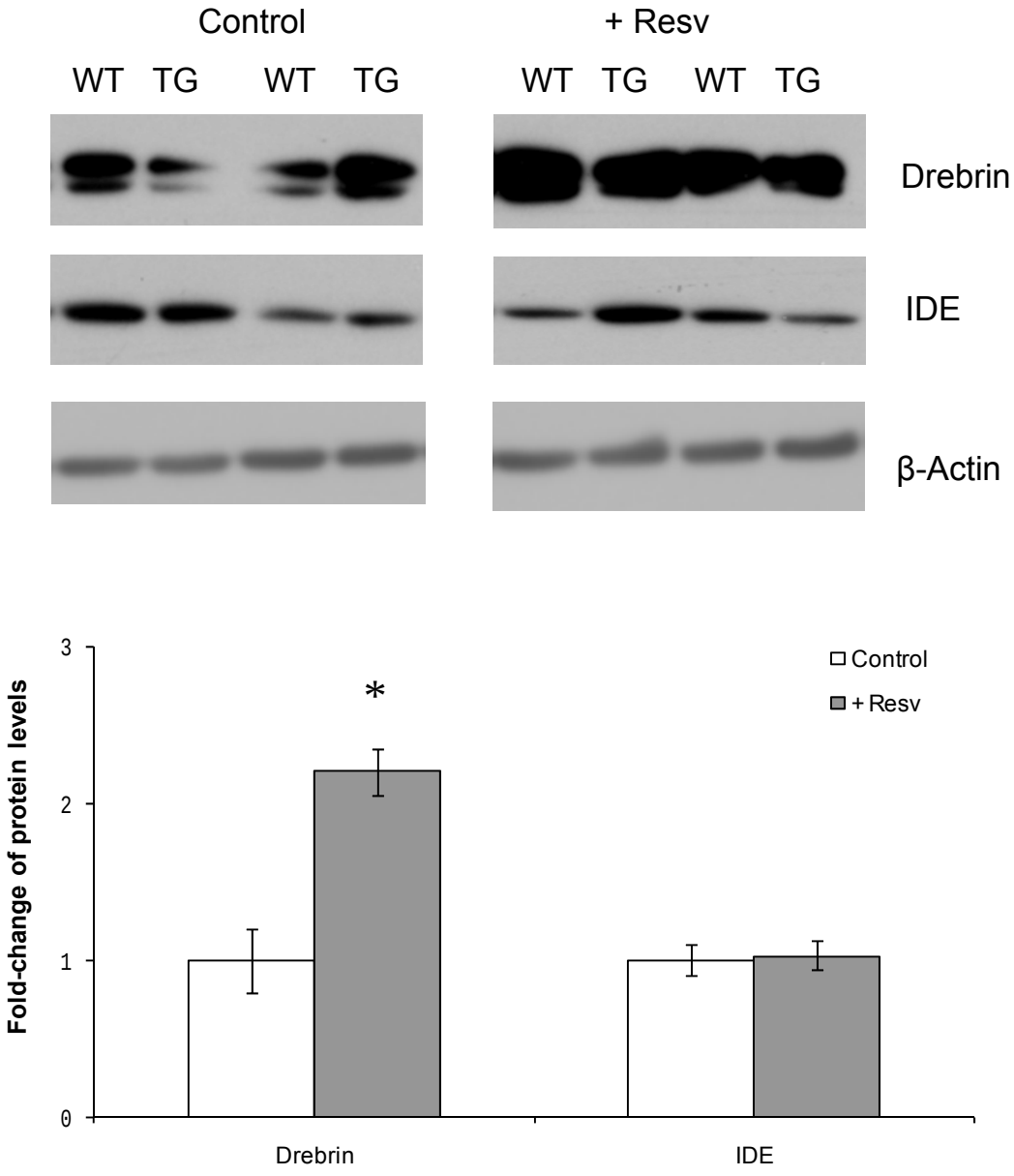

Figure 4.2. Resveratrol feeding significantly increased drebrin levels in both wild-type and transgenic animals (2.2-fold increase; $p < 0.05$, pooled data shown). Resveratrol did not alter insulin degrading enzyme levels in either wild-type or transgenic animals ($p < 0.05$, pooled data shown). Data represent means ± SEM of control (n=9) and resveratrol (n=9) groups.

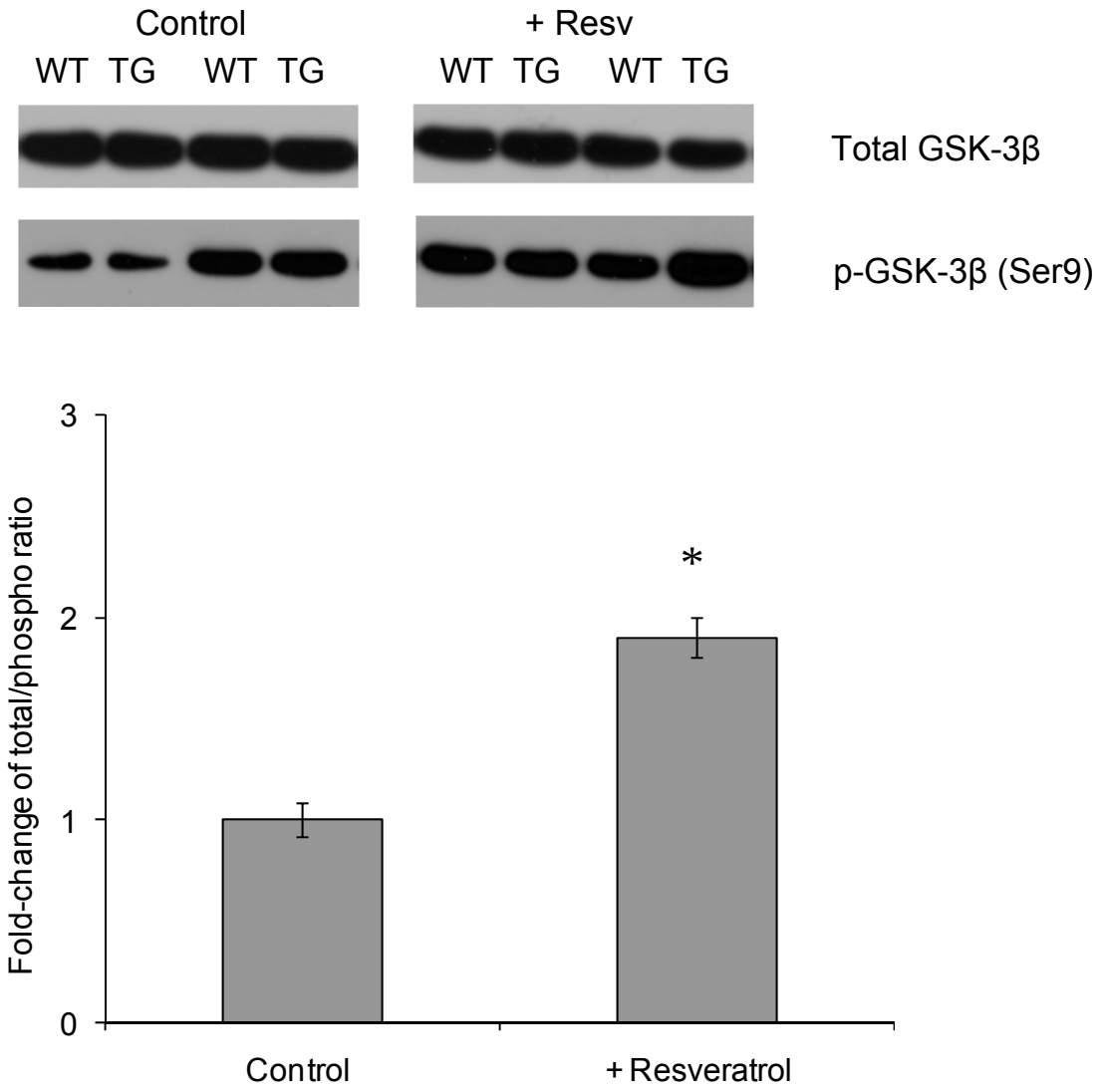

Figure 4.3. Total GSK3-beta and phospho-GSK3 beta (ser9) from brain cortex were measured and levels of phosphorylated GSK3-beta were normalized to total levels of GSK-beta. In both wild-type and transgenic groups, resveratrol significantly decreased GSK-3 activity (1.9-fold increase; $p < 0.05$, pooled data shown). Data represent means ± SEM of control (n=9) and resveratrol (n=9) groups.

in abnormal Aβ production and is hypothesized to be the major mechanism leading to aberrant phosphorylation of tau (33, 34).

Previous studies have shown that GSK co-localizes with neurofibrillary tanges(35) and its activity is increased in the frontal cortex and hippocampus in AD(36, 37). In the context of studies examining apoptosis, cell cycle regulation, and ischemia, *in vitro* data has shown resveratrol's interaction with the phosphatidylinositol-3-kinase/Akt pathway leading to inactivation of GSK3 β by phosphorylation at serine 9 (22, 38, 39). However, to date, no studies to our knowledge have reported the effect of dietary resveratrol in a mammalian system on GSK3 regulation.

In our study, mice fed resveratrol demonstrated 1.9-fold increases in GSK3 β phosphorylation at serine 9 compared to control diet (Figure 4.3). Our resveratrol-fed mice expressed increases in phosphorylation at serine 9 in both wild-type and transgenic groups. Phosphorylation at serine 9 inhibits GSK3 β activity; namely, aberrant phosphorylation of tau a number of sites such as Ser-396 and Ser-404(40). Surprisingly, we detected no differences in total tau or disease-associated phospho-tau Ser-396 and Ser-404(see Figure 4.6). No change in tau phosphorylation could be explained by several factors. First, when acting alone, GSK3 β phosphorylation of tau occurs at very slow rates and increases rapidly when tau is prephosphorylated at separate sites by other protein kinases, such as protein kinase A at Ser-214(41).

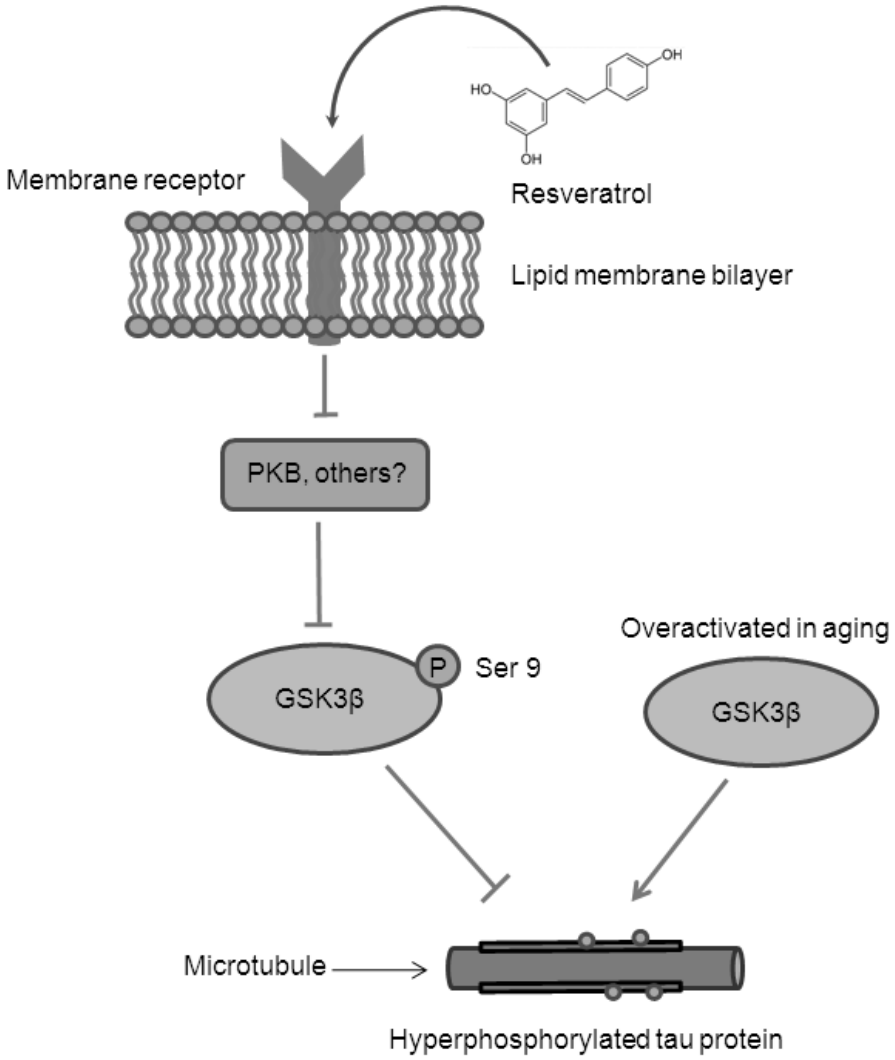

Figure 4.4 Resveratrol inhibits GSK3 beta by phosphorylation at serine 9. A number of proteins including protein kinase B (PKB) and others inhibit GSK3 beta activity by phosphorylating GSK3 beta at serine 9. This phosphorylation event inhibits hyperphosphorylation of the microtubule-associated protein tau which can lead to neurofibrillary tangles, a hallmark of Alzheimer's disease. The figure describes a proposed method where resveratrol directly binds a membrane receptor and inhibits GSK3 beta through inhibiting PKB.

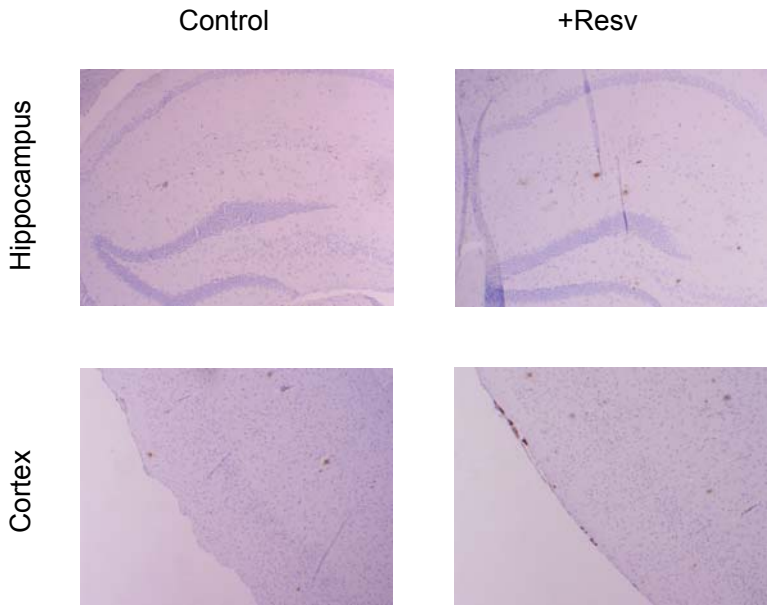

Figure 4.5. Resveratrol did not alter plaque load in APP/PS1 transgenic AD mice. Representative coronal brain sections from AD transgenic mice showing hippocampus and cortex stained with antibody specific for extracellular beta-amyloid.

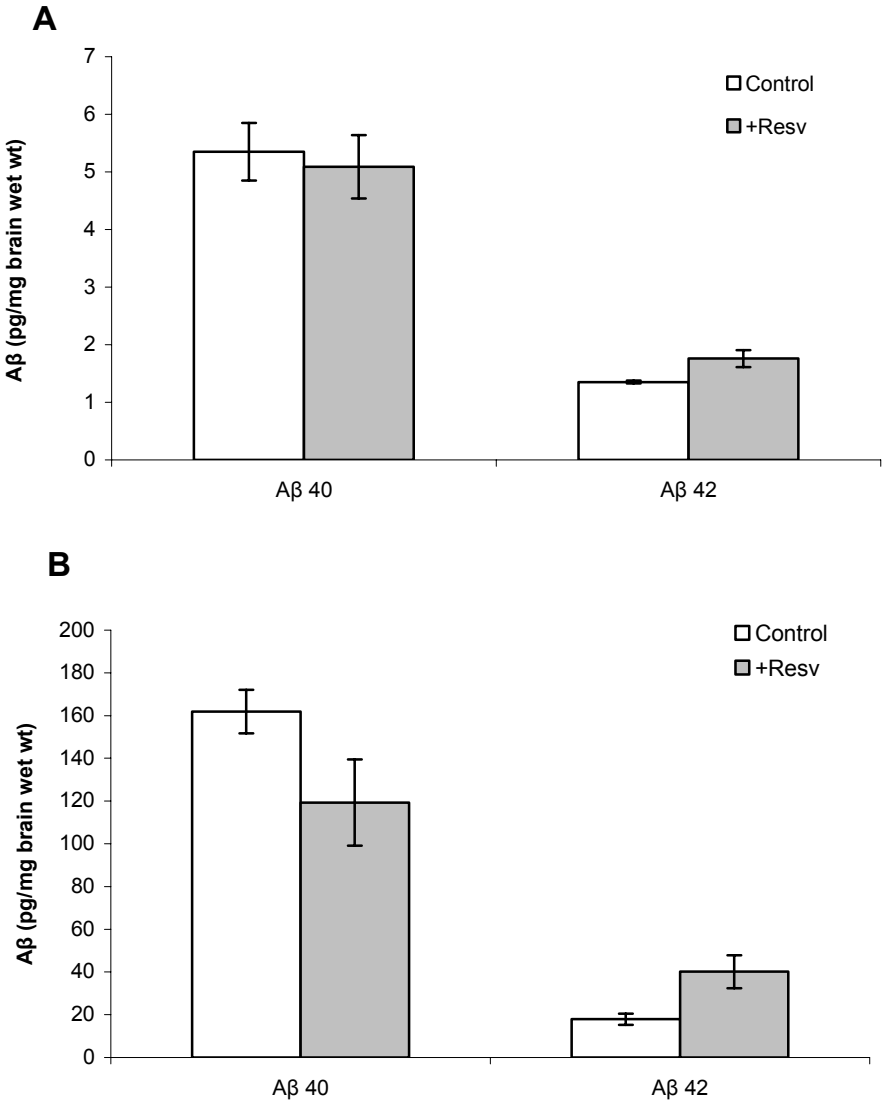

Figure 4.6 *A,* ELISA of Aβ40 and Aβ42 levels in the carbonate soluble fraction of cortex homogenate show no significant differences between dietary groups. *B,* ELISA of Aβ40 and Aβ42 levels in the carbonate insoluble (guanidine-soluble) fraction of cortex homogenate show no significant differences between dietary groups. Data represent means ± SEM of control (n=3) and 0.19% resveratrol (n=6) groups.

Figure 4.7. Total tau and phosphor-tau (Ser 396 and Ser 404) from brain cortex was measured and levels of phosphorlayed tau were normalized to total levels of tau. Resveratrol did not significantly alter levels of tau phosphorylation at sites Ser396 or Ser404.

Table 4.1. Body weight and food intake measurements were recorded on a weekly basis throughout the experiment. Differences in body weight and total food intake were not statistically significant between dietary groups. Data represent means ± SEM of control (n=9) and 0.19% resveratrol (n=9) groups.

	Before treatment[a]		After treatment	
	Control Diet	Resv Diet	Control Diet	Resv Diet
Body Weight	33 ± 0.8	33.8 ± 1	37.5 ± 0.7	39 ± 0.9
Food Intake (g/week)	3.0 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.2 ± 0.2

a - Treatment was 16 weeks dietary resveratrol at 0.19%. Before treatment, all mice consumed standard control diet, AIN93G.

Since resveratrol's inhibition of GSK3 β did not lead to differences in phospho-tau Ser-396 and Ser-404, it is possible that tau was not primed via prephosphorylation by protein kinase A or other kinases. Priming of tau could have conceivably led to increased phospho-tau in non-resveratrol fed animals and increased differences between control and resveratrol diet groups. Second, rapid endogeneous dephosphorylation of normal tau proteins occurs after death; it has been observed that up to 80% of tau immunoreactivity at sites Ser-396 and Ser-404 disappears after a postmortem delay of 2 h at room temperature(42). In the case of our study, however, this remains unlikely as mice were rapidly dissected and brain sections were flash frozen within 5 minutes of sacrifice. Additionally, brain lysates were treated with protease and phosphatase inhibitors. Third, it may be worth noting that our measurement is from the soluble fraction of the brain lysate, and that insoluble tau could represent a different pattern of phosphorylation at Ser-396 and Ser-404. Lastly, other posttranslational modifications of tau such as glycosylation have been shown to play a role in its phosphorylation. For example, deglycosylation of tau surpresses subsequent phosphorylation at Ser-404(43). Regardless, it remains clear that resveratrol's ability to phosphorylate and inactivate the critical target GSK3 β constitutes an important new mechanism underlying resveratrol's neuroprotective effects.

Further, GSK3 remains an important target for a number of other physiological conditions. GSK3 inhibitors have shown beneficial effects for diabetes (increased glucose transport and decreased blood glucose), inflammation (increase of anti-inflammatory mediators and prevention of arthritis), cancer (inhibition of NF-κB), cardiac ischemia (reduction of infarct size), and stem cell

stimulation(44-50). Resveratrol's inhibitory action on GSK3 represents an exciting finding whose effects may extend beyond neuroprotection.

The precise relationship between cerebral Aβ deposition, tau pathology, and clinical AD is not obvious, as several studies have demonstrated amyloid deposition in cognitively normal aged individuals (51-53). A number of pathological studies may indicate that synaptic defects may be more directly related to AD, as post-mortem tissue studies suggest synaptic dysfunction as an early event in AD(54, 55). Substantial losses of postsynaptic proteins such as developmentally regulated brain protein (drebrin) and post-synaptic density protein 95 (PSD-95) have been reported in AD(17, 56, 57).

Drebrin is a dendritic spine protein which plays an important role in synaptic function, losses of which have been reported beyond 70% in a number of separate studies and have been found in subjects with mild cognitive impairment(17, 57, 58). Levels of drebrin are significantly decreased in AD and correlate well with tau pathology(56, 57, 59). Further, losses in drebrin protein in the temporal cortex correlate to decreases in Mini-Mental State Examination and Braak scores, two questionnaire-based tests that predict neuropathologic stage(60). Drebrin has been shown to respond to diet, as its loss has been shown to be exacerbated in AD mice fed high-fat diets low in n-3:n-6 polyunsaturated fatty acid ratio and rescued in AD mice fed DHA(61).

In our study, resveratrol-fed mice exhibited 2.2-fold increases in cortex drebrin (Figure 4.2). Increases were observed regardless of wild-type or transgenic state, with both groups of animals exhibiting increased drebrin when fed

resveratrol. It is worth noting that differences in drebrin levels did not exist between wild type and AD mice on control diet, therefore this transgenic model may come up short in an attempt to mimic this physiological feature of AD. Regardless, a positive change in drebrin levels in the presence and absence of an AD genetic background indicates resveratrol's action on drebrin may be independent of background neuropathic state. As with any protein, drebrin losses can be caused either by decreasing production (transcription or translation) of the protein or increased degradation.

In this case, since drebrin levels did not differ significantly between wild-type and AD groups and were higher in all resveratrol-fed animals, it is likely that drebrin production increased either through increased mRNA transcripts or increased translation of existing transcripts. Increased drebrin mRNA expression has been shown to be inversely correlated to insoluble tau and paired helical fragment tau concentrations which make up neurofibrillary tangles, suggesting that drebrin losses are very closely related to AD pathology(59, 62, 63). Further, as drebrin has demonstrated to be an important predictor in memory function in even mildly impaired subjects, resveratrol intake could lead to improved neuronal health in populations that are otherwise dementia-free. Improvements in drebrin by resveratrol may prove to be an important observation as it describes a direct target and mechanism between resveratrol and neuronal health.

As evidence has indicated an association between between altered APP processing and increased amyloid production and the development of AD (64), a concerted effort has been made in the last decade to develop drugs and

treatments that decrease the production or increase the clearance of Aβ (65).

Transthyretin (TTR) represents at least 20% of the total protein in the cerebrospinal fluid. It is synthesized and secreted by the choroids plexus(66) and has been identified as the main Aβ binding protein in human CSF(67, 68). TTR has been shown to bind and sequester Aβ protein and prevent Aβ aggregation (69). *In vitro*, purified TTR can inhibit Aβ fibrils (70) and in *C. Elegans* has shown to lead to significant reductions in Aβ plaques (71). Further, an inverse relationship has been found between levels of TTR in human cerebrospinal fluid and severity of AD (72). Studies have shown TTR is responsive to dietary compounds, including ginkgo biloba (73) and the long-chain polyunsaturated fatty acid docosahexaenoic acid (DHA) (74). Previous studies showing that resveratrol inhibits TTR-induced cytotoxicity make it a strong candidate for inhibiting TTR *in vivo*(75).

In our study, dietary resveratrol increased levels of cortex TTR protein by 3.8-fold (Figure 4.1). However, increased TTR levels did not translate into decreases in Aβ plaque, and TTR levels were only marginally higher in resveratrol fed AD mice versus control diet AD mice ($p=0.07$, data not shown). Resveratrol-binding sites have been discovered broadly in the rodent brain but appear most concentrated in the choroids plexus where TTR is produced. Further, resveratrol has been shown to be a potent mitogen-activated protein kinase (MAPK) activator, leading to activation of protein kinase A (PKA) or protein kinase C (PKC)(23). PKA and PKC regulate cAMP response element binding protein (CREB) which is suggested to activate downstream genes such as TTR and others involved in memory(76, 77). Altogether, this suggests

that resveratrol could increase TTR expression through binding at the choroids plexus, leading to MAPK, PKA, PKC, and CREB signaling cascades. In this study, it is possible that increases in TTR were not significant enough to translate into measurable differences in Aβ, or that TTR has a limited window of opportunity during Aβ accumulation to sequester and prevent intraneuronal Aβ aggregation. In spite of this, dietary modulation of TTR by resveratrol provides a new putative mechanism whereby resveratrol can exert protective neurological effects.

Insulin degrading enzyme (IDE) is a proteolytic degrading enzyme for insulin and Aβ. IDE is upregulated in response to insulin signaling, and diabetic subjects have decreased insulin signaling and are at increased risk of AD(78). Insulin resistance is associated with decreased cerebral IDE and accumulation of Aβ (79) and increased insulin signaling has shown to prevent Aβ oligomers(80). While resveratrol has been shown to increase insulin signaling and sensitivity (12, 13), our results indicate no changes in levels of IDE when mice were fed dietary resveratrol (Figure 4.2). IDE is a tightly regulated protease and degrades not only insulin and Aβ but also a number of other peptides(81, 82). Further, IDE is much more selective for insulin than for Aβ, and a number of proteins and pathways are involved in maintaining and regulating overall insulin signaling(83). Since IDE degrades soluble Aβ(84), these results are consistent with our ELISA data showing no differences in soluble Aβ levels across dietary groups (Figure 4.2A).

Our study did not find differences in levels of hippocampal or cortex plaque or secreted Aβ protein in cortex between control diet and resveratrol-fed AD mice

(see Figures 4.4 and 4.5). Similarly, Karuppagounder et al have fed resveratrol at the same concentration in the same transgenic AD mouse for 45 days and report no changes hippocampal Aβ plaque, although they report decreased plaque levels in medial cortex (19). A number of studies show that progressive neurodegeneration may occur in AD patients despite removal of plaques, and many cognitively normal humans display Aβ plaques in equivalent densities as Alzheimer's disease individuals(52, 53, 85).

Since the role or neurodegeneration and its relationship with amyloid metabolism and the precise number and order of pathophysiological events that lead to AD are not fully known, nutrients and therapies that interact with targets more upstream than APP metabolism need to be identified. Our findings confirm and extend the role of resveratrol as a neuroprotective nutrient and open the door to a variety of new mechanisms and protein targets whereby resveratrol exerts its action, such as degradation of Aβ (TTR) positive structural and postsynaptic changes (drebrin), and inhibition of taupathology (GSK). These broad pleitropic effects initially appear to be sirt-independent, agreeing with previous studies showing a number of resveratrol's beneficial effects may be different than that of calorie restriction(20, 21).

It is important to note that resveratrol only describes one compound in a broader class called stilbenes, and that present in red wine are a large number of other polyphenols and phytochemicals with potential bioactivity. Health benefits have been reported for stilbenes, anthocyanosides, catechins, proanthocyanidin, as well as other phenolics in red wine (86). Resveratrol, along with many of other compounds, represent an antifungal or antibavterial

mechanism that improves survival of the grapes, and thus exposure to fungus and also geographic factors play a considerable role in the amount of these compounds present in a given sample and can even vary in the same region from one year to the next(87). Thus, reported levels of these different classes of compounds are quite variable.

A study of red wine has shown that stilbenes exist in concentrations from 53-89 ug/g of dry weight grape skin and 17-39 ug/g of dry weight grape skin for resveratrol (88). On average, a 750ml bottle of wine contains 1250 grams of grapes, 30% of which (about 375 grams) is dry weight (89). From the mean resveratrol values described in these data, estimates can be made that, on average, one bottle of wine contains about 10 mg resveratrol. To achieve numbers in a 70 kg human similar to those fed to mice in this study, 98 bottles of wine would need to be consumed per day (range 67 to 153 bottles). If it is assumed that all stilbenes are equally as potent, this number changes from 98 bottles of wine to about 39 bottles per day.

Year harvested, climate, extraction method, and specific grape species all play a role in the amounts of these compounds present in grapes. Further, it is difficult to separate and characterize these compounds using typical chromatographic methods, and technology is currently further being developed to characterize these molecules in a more accurate and rapid manner (90). Practically, it is important to consider that in the case of red wine as well as any other fruit, large numbers of molecules exist with potential bioactivities whose cumulative result can have additive and syngergistic effects on health.

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CHAPTER 5

CONCLUSION

5.1 Summary

We evaluated the presence and specific roles of a number of nutrients in neural development and neurodegeneration. Research has shown the importance of LCPUFA in human breast milk and formula, and worldwide breast milk averages serve as a useful guide for formula infant feeding. Our literature review considered eighty four studies of human breast milk published in English that were indexed by Medline. We included strict criterion to insure mean values for LCPUFA from studies that were well described and used modern chromatographic methods. For instance, studies that included data from only one mother, pooled or banked milk samples, and mothers of preterm infants were excluded. The use of capillary GC columns that fully resolve FA methyl esters were included as opposed to packed columns which cannot resolve DHA and AA and may provide artificially high values. Our calculated mean (±SD) concentration of DHA in breast milk (by wt) is 0.32 ± 0.22% and that of AA is 0.47 ± 0.13%. However, worldwide breast-milk DHA or AA concentrations calculated by our procedure or any other should not be seen as the exclusive criteria for establishing targets for DHA and AA contents in infant formulas. Studies of optimal DHA and AA levels for infant feeding beyond the present mean values are needed, since data have indicated that higher concentrations of DHA in formula support cerebral increases in DHA and induce positive changes in a number of genes(1). Previous studies have also described positive functional effects of these lipids in neurodevelopment as

well as visual acuity(2). Further work should aim to elucidate and describe molecular mechanisms by which DHA and LCPUFA are involved in the function and development of the brain and seek to further define safe and effective levels of feeding in experimental studies.

While *trans* fats have been associated with the harmful effects of partially hydrogenated vegetable oil (PHVO) consumption, more recent studies have shown that these lipids fed in isolation have effects that are markedly different than PHVO(3). Their detection in mammalian tissues, particularly in the brain where some have hypothesized protective mechanisms against their incorporation(4), are an important step in determining their precise physiological significance in the central nervous system and whether dietary recommendations regarding their intake should be made to the public. We describe a sensitive method whereby *trans* 16:1 and 18:1 isomers are detected and quantified in mammalian tissues in normal aged and Alzheimer's disease subjects. It will be important for researchers to recognize that individual isomers of *trans* fatty acids may have differing biological effects, and that the source of *trans* as well as other components of any mixtures containing *trans*, such as PHVO, are important considerations. Future studies aimed at elucidating the specific physiological role of *trans* fats should continue to apply highly sensitive methods to biological tissues in order to study the role of these lipids and their metabolism and in health and disease. Further, studies should test specific isomers alone and in combination to elucidate their specific metabolic fate and consequences.

As oxidation has also been described to be a major factor in aging, the use of

antioxidants, particularly antioxidant rich foods, has been of great research interest. We demonstrate that dietary resveratrol, a grape and red wine polyphenol, has a number of physiological effects in normal aged and transgenic Alzheimer's Disease mice beyond those normally seen in calorie restriction. Further research examining resveratrol's effects on these targets will be important, especially as neurodegenerative animal models whose pathophysiologly more closely reflects that of human conditions are developed. Through binding at the choroid plexus and increasing levels of transthyretin, resveratrol may contribute to increased binding and sequestering of beya amyloid peptides and decreased neuritic plaques. Postsynaptically, resveratrol may provide protection to neurons by maintaining neuronal structure through its ability to increase levels of drebrin, a key protein whose loss in AD is associated with cognitive impairment. Finally, resveratrol's action on glycogen synthase kinase-3 opens the door to studying the role of this molecule in a number of other diseases, such as cancer and diabetes, and provides further explanation for its protective effects in already published studies of the heart(5, 6).

Resveratrol describes only one of a large number of compounds present in grapes and red wine. As this field moves forward, studies elucidating the particular benefit of these polyphenolic and other compounds in isolation or in symphony with each other will be crucial, as these compounds are naturally found in foods in varying concentrations. Already, studies have described the importance of the heterogeneity of these compounds found naturally in foods in fighting neurodegeneration. In particular, one study demonstrated that two distinct polyphenolic combinations from two different grape varieties both

provided neuroprotection in an Alzehiemer's Disease model(7). However, the specific biological mechanisms and molecular events associated with the protection differed between these two grape species. Further studies describing bioavailability and metabolism of these compounds will also be important as researchers begin to tease out specific molecular targets of action. Already, research has shown that a number of compounds in grape seed extract which initially seem to possess only low bioavailability demonstrate improved bioavailability when they are chronically administered over time(8). Clearly, much work in this field remains, as the complex nature and combination of polyphenolic compounds in foods represents a new and exciting area in neurodegeneration research.

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APPENDIX

VALIDATION OF A RAPID, SINGLE TUBLE METHOD FOR PREPARATION OF FATTY ACID METHYL ESTERS FROM INTACT MAMMALIAN SOFT TISSUES

A.1 Introduction

Fatty acid composition and total lipid content of biological soft solid tissues often serve as important markers in a wide range of physiological and nutritional studies. However, conventional fatty acid preparation requires considerable manual manipulation. Lipid extraction typically involves separate steps for sample homogenization and selective organic solvent extraction, followed by a series of acid- or base-catalyzed reactions to produce fatty acid methyl esters (FAME) for gas chromatography (GC) analysis. Since its introduction, the Bligh and Dyer method (BD) (1) of lipid extraction is the most popular means for extracting lipids from biological tissues. The method is a modification of the Folch method (2), uses a more economical solvent/sample ratio, and is more rapid. However, tissue homogenization and lipid extraction must be performed in separate manual steps for each sample, such that the method is burdensome when preparing large numbers of samples.

Garces and Mancha (GM) developed a one-step method for lipid extraction and FAME preparation from fresh plant tissues in order to study the lipid content of several thousand seeds (3). Lipids were extracted using an aqueous reagent mixture consisting of methanol, 2, 2-dimethoxypropane, and concentrated sulfuric acid (85:11:4) and an organic reagent mixture consisting

of heptane and toluene (63:37). Tissue digestion, lipid extraction and fatty acid transmethylation occur in one tube during a two hour incubation at 80°C, upon which the upper phase of a biphasic system contains FAME ready for analysis. Few manipulations and elimination of several steps make this method convenient and employable for lipid analysis of large numbers of samples.

Though the GM method was originally applied to plant tissue, there are no studies that use the GM method for mammalian soft solid tissue. In the present study, a GM method slightly modified for high water content tissue was applied to representative mammalian soft tissue and compared to a classic protocol starting with mechanical homogenization and BD extraction, followed by conventional base catalyzed hydrolysis and FAME preparation with BF_3/methanol. Previous studies have shown that extended incubations in the presence of acid catalysts may generate geometric (cis/trans) isomerization(4), therefore, olive oil was also studied at 1, 2, 4, and 24 hour incubations to test for artifactual isomerization. FAME were analyzed with GC-mass spectrometry and quantified using internal standards.

A.2 Methods

All reagents used were of analytical grade and mixtures were made immediately before use. FAME were prepared from six replicates (100 ± 10 mg) of liver and muscle using both the BD and GM extraction methods. Bovine liver was chosen as a homogenous tissue at the 100 mg scale and canine cerebellum served as representative of a high lipid tissue.

Bligh and Dyer. Frozen tissue was thawed on ice, then homogenized in 1.92 mL distilled water using a Brinkman polytron (Westbury, NY) equipped with a Kinematica homogenizer (Lucerne, Switzerland). 1,2-Diheptadecanoyl-sn-glycero-3-phosphorylcholine (115 µg per 100 mg tissue; 98+% pure, Matreya, Pleasant Gap, PA, USA) was dissolved in chloroform and added as an internal standard. Samples were re-homogenized after addition of 4ml chloroform/methanol (2:1 v/v). The homogenizer probe was rinsed in 3.5 ml chloroform/methanol (2:1 v/v) and the rinse was added to the homogenate. Samples were then vortexed for 30 minutes and centrifuged for 10 minutes at 3500 rpm. Cell debris was removed and the supernatant transferred to clean tubes, and 2.5 ml chloroform and 2.5 ml 1M NaCl were added. Samples were vortexed and centrifuged, after which the top layer of the biphasic system was discarded including a solid precipitate at the interface. The remaining chloroform was evaporated gently under nitrogen.

To prepare FAME, 2mL of 0.5 N methanolic sodium hydroxide was added to samples and heated to 60°C for 5 minutes. Samples were methylated for 10 minutes at 100°C with 14% BF_3 in methanol. After methylation, 2 ml heptane was added and samples were incubated at 100°C for 1 minute and cooled to room temperature. After addition of 2 ml saturated NaCl, samples were centrifuged, after which the upper (organic) layer was transferred to a clean glass tube and gently dried under nitrogen gas. The prepared FAME were suspended in heptane and stored at -80°C until analysis. 5uL droplets of olive oil underwent the same procedure except the homogenization step was replaced by vortexing in the initial step.

Garces and Mancha. Chunks of thawed frozen tissue, about 100 mg each, were placed in 16 x 125 mm ml screw cap test tubes. 1,2-Diheptadecanoyl-sn-glycero-3-phosphorylcholine (115 µg per 100 mg tissue; 98+% pure, Matreya, Pleasant Gap, PA, USA) was was dissolved in chloroform and added to test tubes as an internal standard. Fatty acid extraction and transesterification was performed by the addition of an aqueous and organic mixture.

The aqueous mixture described in the GM originally contains 5% 2,2-dimethoxypropane (DMP) by volume, however, the authors recommend increasing DMP levels when extracting lipids from high lipid and water containing tissues (3). Thus, DMP was added at a concentration of 11% in the aqueous mixture. 1.4 ml of the aqueous mixture (methanol, DMP, and concentrated sulfuric acid 85:11:4 by volume) was added to each sample. Next, 1.6 ml of the organic reagent mixture (heptane and toluene 63:37 by volume) was added, followed by heptane, to bring the total volume up to 5 ml. Samples were capped and sealed with Teflon tape, vortexed for 1 minute, and incubated at 80°C in a shaking water bath for 120 minutes; olive oil was incubated in duplicate for one, two, four, or twenty-four hours. After heating, the samples were cooled to room temperature for 10 minutes and vortexed for 1 minute. To assist in the separation of the organic and aqueous layers, 2ml of saturated NaCl was added, and samples were vortexed and centrifuged for 10 minutes at 3500 rpm. The upper layer was transferred to a clean glass tube and 2mL heptane was added to the sample tube. After additional vortexing and centrifugation, the upper layer of the sample tube was again transferred to the clean glass tube and dried gently under nitrogen. FAME were suspended in heptane and stored at -80°C until analysis.

Fatty acid concentrations were determined using a Hewlett–Packard 5890 series II GC with a SGE BPX70 fused-silica capillary column (25 m × 0.22-mm i.d. × 0.25-μm) with H_2 as a carrier gas. The oven temperature program started at 80°C, increased at 30°C/min to 170°C, was held for 2 min, then ramped at 10°C/min to 240°C for 1 min. Peaks were quantified using methyl heptadecanoate derived from the diheptadecanoyl phosphatidyl choline internal standard, and response factors were applied using an equal weight mixture analyzed separately.

To test for possible artifactual isomerization products, olive oil samples at 1, 2, 4, and 24 hour incubations were analyzed by covalent adduct chemical ionization tandem mass spectrometry (CACI-MS) (5,6), using with *trans* 16:1n-7 and *trans* 18:1n-9 FAME standards (Matreya, Inc..Pleasant Gap, PA USA). A *trans* FAME concentration of 1.0 ng FAME/mg tissue was the lower limit of quantification (LLOQ).

To compare quantities of individual and total fatty acids between the two methods, paired t-tests were calculated in Excel (Microsoft Office 2003, Windows XP Professional) and statistically significant differences are described where $p < 0.05$.

A.3 Results

Table A.1 presents the total lipid weights extracted from liver and brain. Compared to BD, GM extracted a higher concentration of total FAME for brain.

As expected, the lipid concentration was higher for brain than for liver in both methods.

Table A.2 represents the total long chain ($C_{>18}$) polyunsaturated fatty acids extracted from liver and brain. Compared to BD, GM extracted a higher concentration of total LCPUFA from liver and brain.

Figure A.1 is a photograph of six tubes showing BD and GM for liver and brain tissues side by side. The bottom layer of the GM tubes is clear apart from the cell debris settled at the floor of the tube, similar to the BD method, providing visual evidence that GM digests tissue and frees lipids for methylation without physical tissue disruption.

Figure A.2 shows the fatty acid concentration for liver (A) and brain (B) by saturation. For liver, the two methods extracted comparable concentrations of saturated fatty acids, whereas BD extracted more monounsaturates and GM extracted more polyunsaturates. For brain, GM extracted more saturates, monounsaturates, and polyunsaturates. In both cases, the percentage of each category of saturation appears to follow a similar trend, where saturates, monounsaturates, and polyunsaturates represent about 43-44%, 11-13%, and 44-46% of total lipid for liver and 44%, 36-39%, and 17-19% for brain, respectively.

Figure A.3 shows the fatty acid concentration for liver (A) and brain (B) by omega series. For liver, the two methods extracted comparable concentrations

Table A.1. Total FAME extracted from liver and brain for GM and BD (ug FAME/mg start tissue).

Total FAME extracted per tissue (µg FAME/mg tissue)
(Mean ± SD, n=6)

	Liver	Brain
GM	39.6 ± 0.9	55.2 ± 3.5*
BD	39.4 ± 1.8	36.8 ± 2.6

*denotes higher concentration ($p<0.05$)

Table A.2. Total LCPUFA extracted from liver and brain for GM and BD (ug FAME/mg start tissue).

LCPUFA extracted per tissue (µg FAME/mg tissue)
(Mean ± SD, n=6)

	Liver	Brain
GM	12.6 ± 0.6*	10.2 ± 0.9*
BD	11.6 ± 0.5	5.8 ± 0.4

*denotes higher concentration ($p<0.05$)

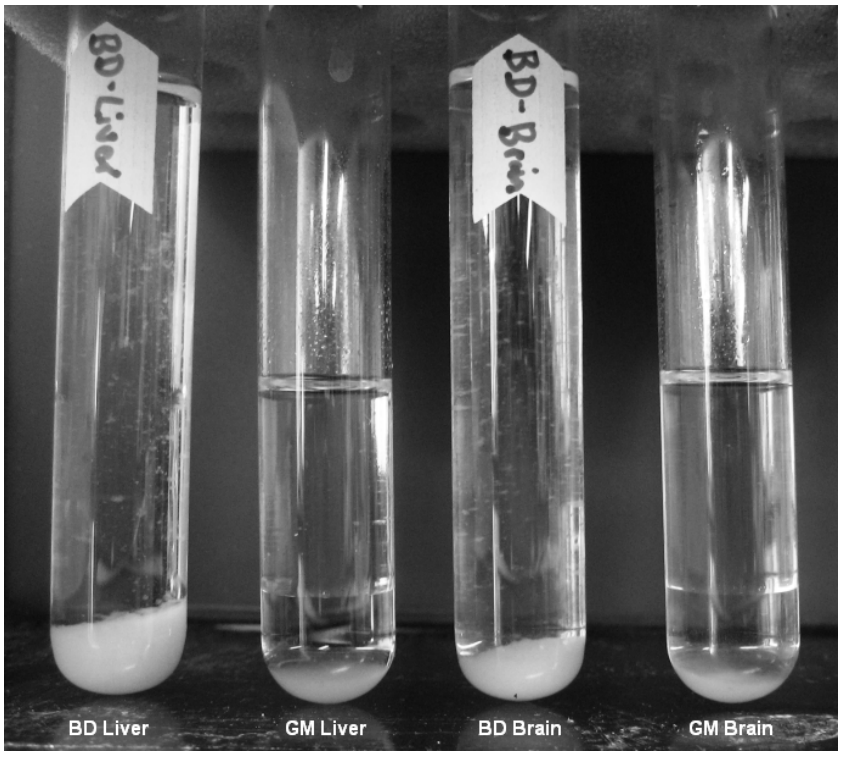

Figure A.1. Cell homogenates, one of each from liver and brain for BD versus GM preparations. The GM tubes are shown post-incubation and vortexing after their contents have settled. The BD tubes are shown after mechanical homogenization, vortexing, and settling before centrifugation.

of n-6, whereas BD extracted more n-7 and GM extracted more n-9 and n-3. For brain, GM extracted more n-9, n-6, and n-3, with no significant differences between n-7 levels. For liver, the percentage of each category of omega series appears to follow a similar trend in both methods where n-9, n-7, n-6, and n-3 represent 30-36%, 3-6%, 51-53%, and 10-11% respectively. For brain, the percentage of each category of omega series are also similar between methods, where n-9, n-7, n-6, and n-3 represent 56%, 12-17%, 19%, and 8-13% respectively.

Table A.3 shows the individual fatty acid concentrations (ng FAME/mg tissue) for liver and muscle. Of the 33 fatty acids detected for the liver, 14 are higher in GM and 7 are higher in BD. Of the 24 fatty acids detected for the brain, 19 are higher in GM and 2 are higher in BD. 18 of the fatty acids for which differences were detected between methods were long-chain ($C_{>18}$) polyunsaturated fatty acids; in all of these, GM extracted a higher concentration of LCPUFA than BD.

The methods were also compared for artifactual isomerization of monoenic fatty acids using olive oil. Neither method produced detectable artifactual isomerization with as much as a 24 hour incubation above the LLOQ.

A.4 Discussion

Garces and Mancha (GM) extracted significantly higher concentrations of total fatty acids for brain but not liver compared to Bligh and Dyer (BD), as shown in

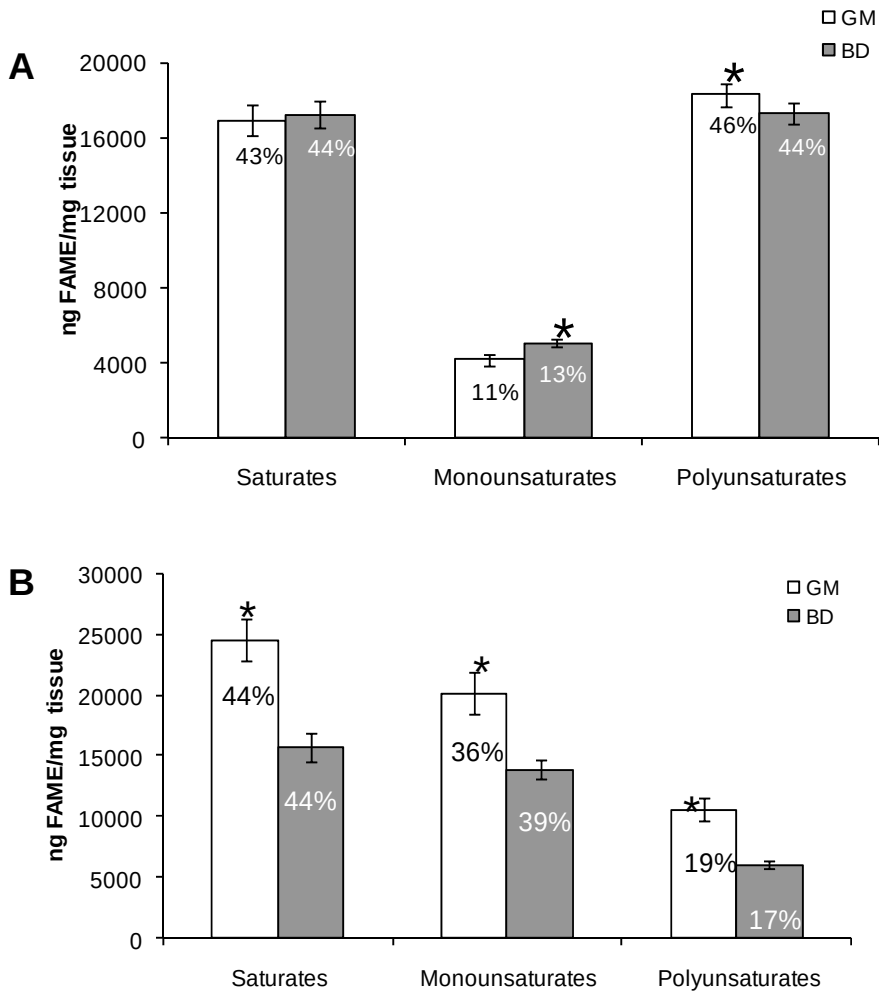

Figure A.2. Total FAME extracted from liver (A) and brain (B) for GM and BD (ug FAME/mg start tissue) by saturation. Percent values within bars represent percent total of each category extracted per method by saturation. Asterisks represent significantly higher concentration of FAME extracted.

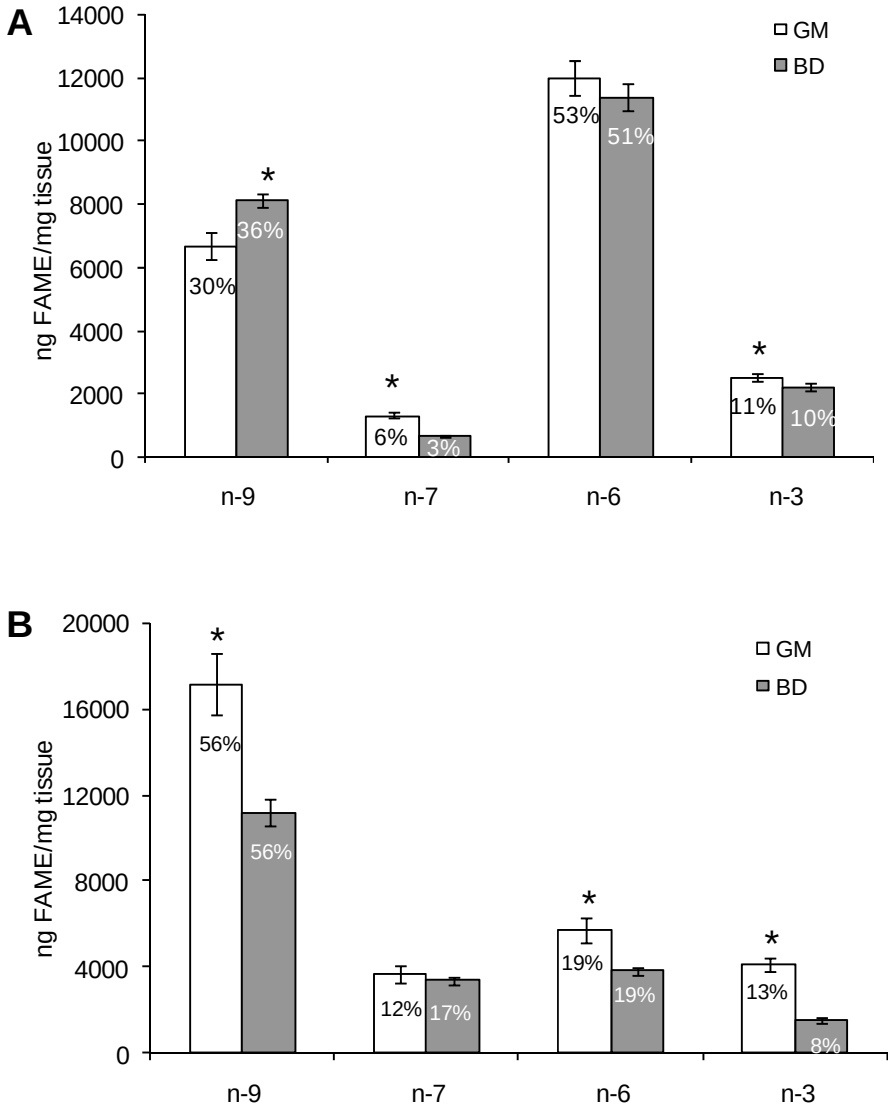

Figure A.3. Total FAME extracted from liver (A) and brain (B) for GM and BD (ug FAME/mg start tissue) by omega series. Percent values within bars represent percent total of each category extracted per method by omega series. Asterisks represent significantly higher concentration of FAME extracted.

Table A.3. Individual FAME extracted from liver and brain for GM and BD (values expressed as ng FAME/mg tissue ± SD, n=6 per tissue per method). Asterisks represent significantly higher concentration of FAME.

	GM Liver				BD Liver			
14:0	280.6	±	16.7		277.8	±	12.2	
B15:0	29.0	±	2.7	*	13.1	±	2.0	
15:0	95.1	±	3.8		123.1	±	15.1	*
16:0	4732.6	±	238.1		4943.3	±	301.7	
B17:0	119.3	±	6.3	*	18.1	±	1.5	
18:0	11283.7	±	529.0		11673.8	±	350.0	
20:0	73.3	±	3.9		69.4	±	3.8	
22:0	120.8	±	4.1		5.0	±	0.5	
23:0	26.2	±	1.6	*	ND	±	0	
24:0	163.2	±	6.1	*	116.8	±	9.1	
14:1n-9	18.1	±	0.8		36.7	±	4.8	*
16:1n-9	109.8	±	9.7		136.4	±	10.8	*
17:1n-9	179.5	±	14.2	*	62.9	±	5.7	
18:1n-9	2507.0	±	224.7		4142.4	±	143.5	*
20:1n-9	22.1	±	2.2		24.8	±	2.3	*
20:2n-9	41.6	±	1.7	*	28.4	±	2.2	
20:3n-9	3704.7	±	172.5		3681.8	±	64.0	
22:3n-9	23.4	±	1.6	*	ND	±	0	
24:1n-9	67.6	±	4.2	*	ND	±	0	
16:1n-7	65.9	±	2.6		268.9	±	13.3	*
18:1n-7	1208.6	±	90.7	*	392.0	±	30.7	
22:3n-7	32.9	±	2.8	*	ND	±	0	
18:2n-6	5416.5	±	242.1		5441.5	±	112.7	
CLA	5.2	±	0.5		7.1	±	0.7	
18:3n-6	183.4	±	9.5		186.5	±	8.9	
20:4n-6	4022.9	±	178.0		3879.9	±	184.6	
22:4n-6	2335.9	±	112.1		1836.8	±	131.4	
22:5n-6	49.1	±	2.1	*	44.6	±	4.9	
18:3n-3	110.3	±	5.8		110.6	±	3.2	*
20:4n-3	105.6	±	3.8		76.5	±	3.1	
20:5n-3	176.2	±	13.5	*	123.9	±	10.5	
22:5n-3	1637.9	±	76.1	*	1521.4	±	88.5	
22:6n-3	462.4	±	21.0	*	386.5	±	28.8	

	GM Brain				BD Brain			
14:0	167.7	±	9.6	*	107.8	±	3.5	
16:0	8883.3	±	526.7	*	7172.3	±	503.8	
18:0	11993.2	±	817.0	*	8045.0	±	609.4	
20:0	437.7	±	33.6	*	66.7	±	6.5	
22:0	497.3	±	44.4	*	31.3	±	2.4	
24:0	2538.9	±	273.3	*	237.4	±	23.0	
16:1n-9	183.8	±	19.2	*	116.6	±	11.6	
18:1n-9	12064.7	±	886.0	*	9539.7	±	510.3	
20:1n-9	688.5	±	38.6		715.8	±	54.2	
20:2n-9	189.1	±	14.1		175.4	±	11.2	
20:3n-9	502.9	±	45.1		522.4	±	37.3	
22:1n-9	221.2	±	24.9	*	131.2	±	7.7	
24:1n-9	3309.2	±	376.5	*	ND	±	0	
16:1n-7	351.6	±	32.6	*	244.2	±	10.4	
18:1n-7	2122.1	±	273.8		2646.1	±	163.7	
20:1n-7	345.3	±	23.5		373.6	±	15.9	*
22:1n-7	239.8	±	20.8	*	97.3	±	8.5	*
24:1n-7	625.3	±	65.3	*	ND	±	0	
18:2n-6	296.3	±	32.9	*	262.5	±	15.6	
20:4n-6	3604.2	±	395.5	*	2374.8	±	149.4	
22:4n-6	1638.6	±	148.7	*	1047.6	±	27.8	
24:4n-6	201.2	±	18.4	*	127.9	±	9.2	
22:5n-3	185.8	±	21.4	*	105.3	±	6.6	
22:6n-3	3922.6	±	294.0	*	1426.9	±	123.1	

Table A.1. For brain, GM extracted 42% more lipid than BD. As liver is a highly homogeneous tissue at the 100 mg sample size, the variability associated with liver lipids was smaller than cerebellum, a highly heterogeneous tissue.

Though GM extracted more lipids from brain than BD but similar levels for liver, the overall percentage of FAME extracted by degree of saturation and omega series remain comparable (Figures A.2 and A.3). This shows that, overall, there is no substantial selection bias or favorability based on saturation or omega series between the two methods; GM simply extracts more lipids from brain, a fattier tissue, than liver. Studies with samples containing <2% lipid have shown the Bligh and Dyer method to be very effective and reliable(7,8).

However, in examining fish muscle with exogenously added fish oil, Iverson et al. have demonstrated the reduced efficiency of the Bligh and Dyer method compared to the Folch method(9). In their study, underestimation of lipid content by the Bligh and Dyer method increased significantly with increasing lipid content; in fact, in their highest lipid samples, lipid content was underestimated by up to 50% using the Bligh and Dyer method. The authors hypothesize that reductions in the final lipid yield by BD may be partially explained by fractions of the organic phase absorbed by the tissue which contains an equal lipid content as the recovered organic phase, thus leading to overall loss of lipids(8).

Similarly, since BD extracts native lipids as distinct covalent molecules, lipid losses are expected for fatty acids that are covalently linked to otherwise charged or very polar compounds, such as proteins in undigested tissue debris which remain with the aqueous phase and would usually be discarded. Specifically, studies have described selective readsorption of acidic phospholipids bound to protein in BD leading to lower yields(10), and inclusion of acid in the solvent system has been shown to recover adsorbed phospholipids(11).

GM also extracted significantly higher concentrations of polyunsaturated fatty acids in both brain and muscle. Our extraction of FAME derived from BD was done with hexane or heptane, as is common in most labs, and may select against PUFA FAME. PUFAs have higher solubility in slightly polar or hydrophilic solvents(12). The presence of toluene in the organic phase in GM may partially explain improved PUFA yields in GM as it is slightly hydrophilic compared to hexane or heptane.

The key feature of the BD lipid extraction method is careful adjustment of the solvent mixture (water, chloroform, methanol) to achieve a monophasic mixture, where the proportions are governed by the chloroform-methanol-water ternary phase diagram. The amount of water initially added to the homogenate must be adjusted based on the water content of the tissue in question to achieve a monophasic system. Chloroform or water is then added later to convert to a biphasic system wherein the lipid is contained in the organic phase.

In this respect, BD is initially a dissolution, followed by an extraction. BD, then, yields all lipid classes which can be analyzed by, for instance, electrospray ionization mass spectrometry according to methods now termed “lipidomics”, or conventional HPLC or TLC to purify lipid classes prior to FAME analysis.

In contrast, GM yields exclusively FAME for immediate analysis, and cannot be used for subsequent lipidomics analysis. GM proceeds strictly as a biphasic system in which homogenization, saponification, methylation, and extraction are accomplished in one tube by chemical means, with vigorous or gentle shaking as the only mechanical agitation. The first three steps take place in the aqueous phase, and as FAME are synthesized they transfer to the organic (top) phase. Digestion of tissue, fatty acid hydrolysis, and acid catalyzed methylation proceed simultaneously by the action of H_2SO_4. Thus, GM may have the advantage of extracting bound fatty acids that BD does. Further, less pipetting/manual transfer of phases in GM may result in less sample loss and higher yields than BD. However, GM cannot be adapted in any obvious way for analyses that require saponifiable lipid classes to be preserved.

Non-saponifiable lipids are extracted into the organic phase with GM. They can obscure important FAME in chromatographic traces when tissue contains significant quantities. In our hands, chromatograms of a variety of samples have shown squalene and carotenoids that would be problematic for GC-FID analyses. We routinely perform molecular identification with GC/MS/MS, and can perform quantitative analysis with MS/MS with proper choice of methods, and thus have not found this to be a major disadvantage.

Physical homogenization of samples and the need to break emulsions by centrifugation are two steps that are expensive to automate. Separate steps for sample homogenization, lipid extraction, and FAME preparation present time and scale-limiting barriers using conventional methods. Large clinical studies that examine the relationship between fatty acids and health outcomes demand efficient, streamlined methodology. Lepage and Roy (13) presented a one-step method whereby plasma fatty acids are directly transesterified in one hour with high recoveries. Masood et al. (14) adapted this technique to demonstrate an automated robotic method to perform FAME analysis on a large number of research samples from clinical trials. GM is similarly amenable for preparation of FAME from mammalian soft solid tissue for high-throughput analyses.

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